Transcriptional regulation of the central carbohydrate metabolism and synthesis of trehalose in the hyperthermophilic crenarchaeote *Thermoproteus tenax*

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Meinen Eltern -

In Liebe und Dankbarkeit

Was wir wissen, ist ein Tropfen.

Was wir nicht wissen - ein Ozean.

Isaac Newton

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1 INTRODUCTION

Evolution of life led to three major domains of living organisms: the Eucarya and two distinct prokaryotic domains, the Bacteria and Archaea. Originally, the domain of the Archaea was identified by Carl Woese and Geoge E. Fox (Woese and Fox, 1977; Woese *et al.*, 1990) as being the third major line of life based on 16S rRNA sequence analyses.

The Archaea are further divided into the kingdoms of the Euryarchaeota, Crenarchaeota and the Korarchaeota (see fig. 1.1), whereas latter represents a group of mainly uncultivated organisms (Barns *et al.*, 1996). An additional separated branch within the Archaea, the Nanoarchaeota, has recently been discovered (Huber *et al.*, 2002).

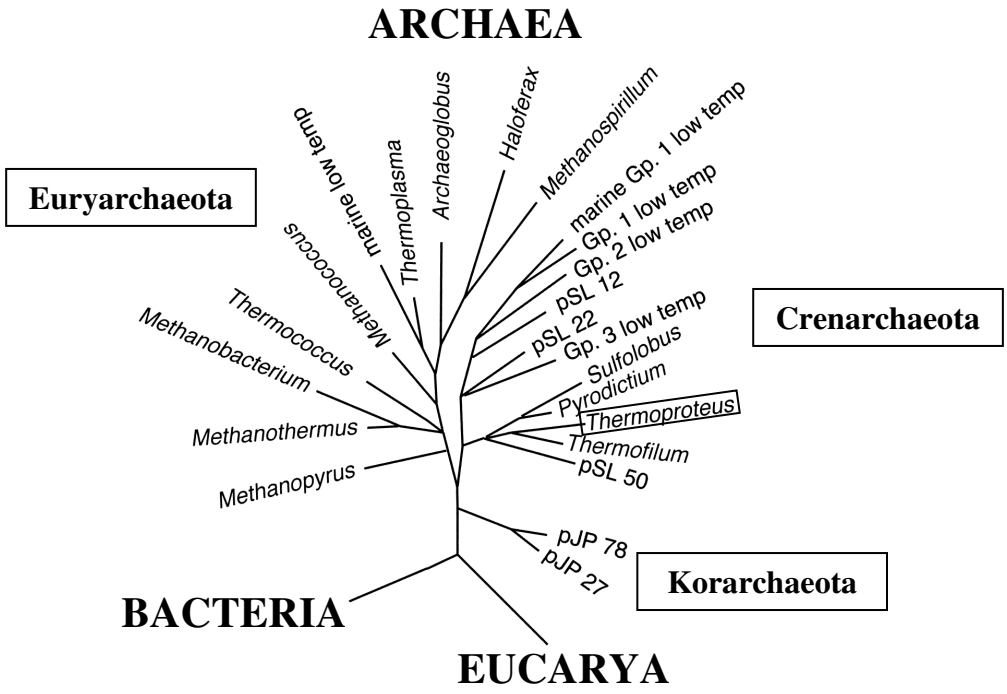


Fig. 1.1 **Phylogenetic tree of the Archaea based on 16S rRNA sequence analyses.** (modified, Pace *et al.*, 1997)

Adaptation and specialisation to harsh environments represent hallmarks of Archaea. Some species survive and thrive at temperatures over 100°C, cold temperatures down to 0°C, in extremely alkaline or acidic waters (pH 1-2) or extremely saline environments (> 30% (w/v) salts). With respect to their extreme environments, those Archaea are also termed "extremophiles" and have been isolated from rift vents in the deep sea (black smoker),

geysers, hot acidic springs and sulphuric waters, salt lakes and the Dead Sea, but also from digestive tracts of cows or termites and anaerobic muds of marshes.
The kingdom of the Crenarchaeota mainly includes hyperthermophilic and thermoacidophilic organisms, whereas the Euryarchaeota represent a more divers group including methanogenic, halophilic and hyperthermophilic species.
However, recent environmental studies revealed that Archaea are in no way restricted to extreme environments, but much more widespread in ordinary habitats than previously thought (De Long and Pace, 2001).
The adaptation strategies of Archaea living in extreme environments became a matter of great interest and excited scientists into unravelling the molecular mechanisms, which are also of great interest for biotechnological applications, for example gaining heat-stable proteins. Although Archaea have been studied in detail, information about the biochemical and physiological features of archaeal metabolism, as well as their information processing pathways is still rather scarce in comparison to Bacteria and Eucarya.

Further insights into the domain of the Archaea is given by a number of completed archaeal genome sequencing projects (about 24 euryarchaeal and 11 crenarchaeal genomes; http://archaea.ucsc.edu/), which have shown that Archaea represent a chimaera of bacterial and eukaryotic features. Their core metabolic functions resemble those of Bacteria, whereas their information processing functions are distinctly eukaryotic (Koonin and Galperin, 2003; Makarova and Koonin 2003; Allers and Mevarech, 2005).
Recent studies on archaeal genomes and reconstruction of archaeal metabolism revealed a wide diversity of archaeal physiology (Koonin and Galperin, 2003). Focusing on the central carbon metabolic pathways, it was shown that a large number of heterotrophic Archaea represent sugar metabolising species. Although glycolytic pathways are conserved in Bacteria and Eucarya, comparative studies in Archaea revealed that carbohydrates are metabolised by variants of the classical Embden-Meyerhof-Parnas (EMP) and the Entner-Doudoroff (ED) pathway, which are characterised by a great variety of novel archaeal enzymes (Siebers and Schönheit, 2005; van der Oost and Siebers, 2007), the presence of which can best be explained by independent, convergent evolution (Verhees *et al*., 2003).
Although the complexity and modifications of archaeal central carbohydrate metabolism (CCM) are well established not much is known about its regulation.
To fill this gap, respective analyses were performed in the course of the present work for *Thermoproteus tenax*.

Thermoproteus tenax is a hyperthermophile belonging to the kingdom of the Crenarchaeota and was the first hyperthermophilic Archaeum described. The *T. tenax* strain Kra1 was originally isolated from a solfatare in Iceland (Zillig *et al.,* 1981; Fischer *et al.*, 1983; see fig. 1.2). *T. tenax* is a rod-shaped, strictly anaerobic, sulphur reducing organism with optimal growth at 86°C and pH 5.6. The organism is able to grow chemolithoautotrophically on carbon dioxide and hydrogen as well as chemoorganoheterotrophically in the presence of various organic substrates, e.g. glucose, starch, amylose, malate, glycerol, glycerate or ethanol (Zillig *et al.*, 1981; Fischer *et al.*, 1983). It has been suggested that under autotrophic growth conditions CO_2 fixation functions via the reductive citric acid cycle (CAC), as described for the closely related *Thermoproteus neutrophilus* (Beh *et al.*, 1993).

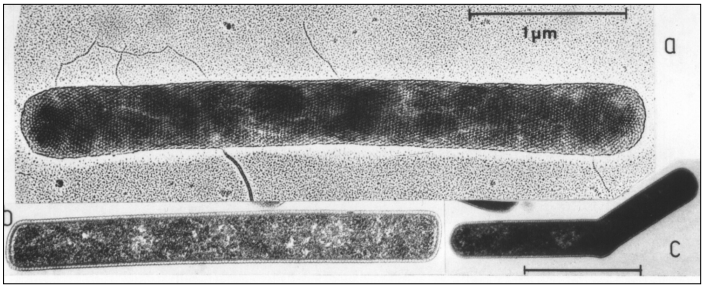


Fig. 1.2 **Electron micrograph of *Thermoproteus tenax* Kra1.** (Zillig *et al.*, 1981)

T. tenax is the only currently known Archaeum that uses two different pathways for the degradation of glucose in parallel - the reversible Embden-Meyerhof-Parnas (EMP) pathway and the branched Entner-Doudoroff (ED) pathway, both of which represent modified versions of the classical pathways known from Bacteria and Eucarya (Siebers and Hensel, 1993; Selig *et al.*, 1997; Siebers *et al.*, 1997 Siebers *et al.*, 2004; Ahmed *et al.*, 2005). Its additional versatility in catabolising a variety of organic substrates and its ability to grow also autotrophically make *T. tenax* an ideal object to study the regulation of central carbohydrate metabolism (CCM) in response to the offered carbon source (glycolytic/gluconeogentic switch of metabolism).

In contrast to the classical version of the EMP pathway known from Bacteria and Eucarya, the *T. tenax* EMP variant is characterised by an ATP-dependent hexokinase (ATP-HK) (Dörr *et al.*, 2003) and a reversible pyrophosphate-dependent phosphofructokinase (PP_i-PFK; Siebers *et al*., 1997), which replaces the antagonistic enzyme couple ATP-dependent PFK and fructose bisphosphatase (FBPA). Both enzymes do not show notable regulatory properties and

therefore, the two control points described for the classical version of this pathway are missing in *T. tenax*.

At the level of glyceraldehyde 3-phosphate (GAP), three different GAP-converting enzymes are found: The highly allosteric non-phosphorylating glyceraldehyde 3-phosphate (GAP) dehydrogenase (GAPN), the classical phosphorylating GAPDH (Brunner *et al.*, 1998, 2001) and the non-phosphorylating ferredoxin-dependent GAP oxidoreductase (GAPOR). Furthermore, phosphoenlpyruvate (PEP)/pyruvate conversion in *T. tenax* is characterised by a catabolic pyruvate kinase (PK) with only a very low regulatory potential (Schramm *et al.*, 2000), the reversible pyruvate phosphate dikinase (PPDK), which catalyses the interconversion of PEP and pyruvate, but rather represents a catabolic enzyme, and the anabolic phosphoenolpyruvate synthetase (PEPS; Tjaden *et al.*, 2006).

The alternative route for glucose degradation occurs via the branched Entner-Doudoroff (ED) pathway (Ahmed *et al.*, 2005). Comparative genomic approaches and *in vitro* reconstruction experiments revealed the presence of the non- as well as the semi-phosphorylative ED pathway (Ahmed *et al.*, 2005), the latter was long time thought to be typical only for halophiles. The *T. tenax* 2-keto-3-deoxy-(phospho)gluconate aldolase (KD(P)GA), the key enzyme of the non-phosphorylative ED variant, turned out to be a bifunctional enzyme utilising KDG as well as KDPG and therefore represents a key enzyme in both ED branches. The glycerate kinase is the characteristic enzyme of the semi-phosphorylative ED variant and catalyses the phosphorylation of glycerate to 2-phosphoglycerate.

The pathway parallelism characterised by the reversible EMP, which was shown to be the main route for glucose degradation in *T. tenax* (Siebers *et al.*, 1997), and two branches of the ED pathway raises questions about the physiological function, especially of the two ED branches in *T. tenax*.

In *T. tenax*, the operation of a reversible citric acid cycle (CAC) is suggested, involved in the complete oxidation of pyruvate (oxidative direction under heterotrophic growth conditions; Selig and Schönheit, 1994) as well as for carbon dioxide fixation (reductive direction, autotrophic growth conditions; Siebers *et al.*, 2004). Therefore a coordinated regulation of the gene expression of the key enzymes of the CAC is supposable.

T. tenax also possesses pathways for the synthesis and degradation of the carbon storage compound glycogen, which was identified previously (König *et al.*, 1982) as well as the compatible solute trehalose.
Recent studies revealed that the classical oxidative pentose phosphate pathway common in Bacteria and Eucarya seems to be generally absent in Archaea. It appears that pentoses for anabolic purposes in Archaea are provided by the non-oxidative pentose phosphate (NOPP) pathway and/or the reversed ribulose monophosphate (RuMP) pathway (Verhees, 2003; Soderberg, 2005). The *T. tenax* genome harbours all homologs expected for an active RuMP pathway (Van der Oost and Siebers, 2007).

A prerequisite to study the biochemical and genetic mechanisms that select the various pathways and regulate the carbon flux through them is the availability of the *T. tenax* genome sequence. The *T. tenax* genome was deciphered in collaboration with Dr. H.-P. Klenk (e.gene, Feldafing, Germany) and Prof. Dr. S.C. Schuster (MPI für Entwicklungsbiologie, Tübingen, Germany / Pennsylvania State University, USA).
The sequence information has been used for the reconstruction of the CCM of *T. tenax*, including the above desribed pathways (Siebers *et al.*, 2004; see fig. 1.3). This information of the reconstructed CCM of *T. tenax* was the basis for the design of the focused CCM cDNA microarray.

Regulation of archaeal metabolism is supposed to be executed at all levels, including DNA, RNA and protein level, as demonstrated for Bacteria and Eucarya. However, for the well-characterised EMP pathway, regulation at protein (enzyme) level is rare compared to the respective pathways in Bacteria and Eucarya (Verhees *et al.*, 2003; Siebers and Schönheit, 2005; Van der Oost and Siebers, 2007) and regulation at translational level has not yet been demonstrated in Archaea. Therefore it has been supposed that an important site of regulation of archaeal metabolism appears to be at transcriptional level.

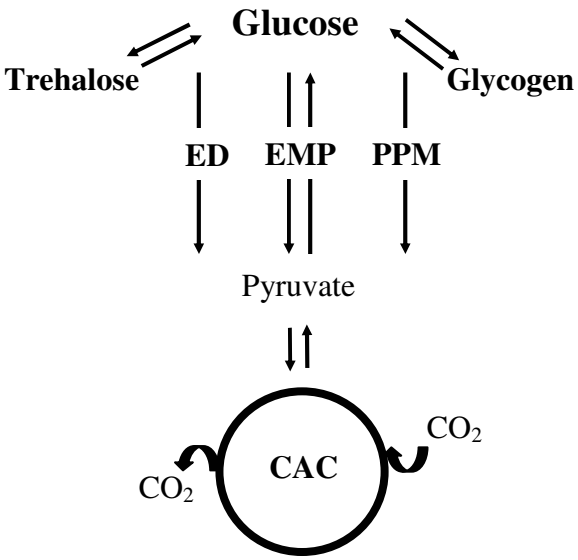


Fig. 1.3 **Scheme of the pathways involved in the central carbohydrate metabolism (CCM) of *T. tenax*.** ED – Entner-Doudoroff pathway, EMP – Embden-Meyerhof-Parnas pathway, PPM- pentose phosphate metabolism, CAC- citric acid cycle

DNA microarrays

In an era of genome sequencing and consequently, the discovery of thousands of genes, it is essential to possess a technique that allows exploration of the whole genome.

In the last decade, DNA microarrays became a powerful tool for the investigation of many biological processes (Brown and Botstein, 1999; Khan *et al.*, 1999; Eisen and Brown, 1999; Kurella *et al.*, 2001). Microarrays permit comprehensive surveys of gene expression in a highly parallel manner. It is possible to monitor gene expression for thousands of genes (expression/transcriptional profiling) simultaneously, e.g. under different growth conditions.

Microarrays have especially been used in human medical research, e.g. for characterisation of various diseases. However, several studies have also been performed with, e.g. yeast (Jia *et al.*, 2000) or Bacteria like *Escherichia coli* (Khodursky *et al.*, 2000; Oh *et al.*, 2000) or *Bacillus subtilis* (Ye *et al.*, 2000). Recently, whole genome transcriptional profiling has also been performed for some archaeal species, e.g. *Haloferax volcanii* (Zaigler *et al.*, 2003), *Pyrococcus furiosus* (Schut *et al.*, 2003), and *Sulfolobus solfataricus* (Snijders *et al.*, 2006) in order to investigate their metabolism.

A microarray represents an arrangement of hundreds to thousands nucleic acid species (DNA) attached to a solid surface (glass or plastic).

The main principle of the microarray technique is the parallel hybridisation of a mixture of labelled nucleic acids (termed as targets) with the thousands of individual nucleic acid species (termed as probes) contained on the microarray. Probes can be identified by their spatial position ("spot") on the array.
Generally, two types of microarrays are used:

- **cDNA (oligonucleotide) microarrays** contain probes with a length of approximately 500-5,000 bases. The probes are spotted onto the slides using robotics. Arrays are mostly used in two-colour experiments.

- **High-density, synthetic oligonucleotide-based DNA arrays**, e.g. Affymetrix, can have a density of up to 400,000 probes per slide, however probe length is restricted to about 20-80 nucleotides. Probes are synthesised *in situ* directly onto the surface of the slide. Hybridistaion is performed with only one labelled target.

In this study the construction of a cDNA microarray is documented, comprising 111 probes of 105 genes involved in the different pathways of the CCM in *T. tenax* (see fig. 1.3). This focussed *T. tenax* CCM cDNA microarray was used to monitor changes of gene expression in response to different carbon sources (glucose and CO_2), to find out more about the regulatory processes that are required to control the carbon flux as well as the glycolytic/gluconeogenic switch of carbon metabolism in *T. tenax*.

The main steps of microarray analyses include probe generation via PCR amplification and subsequent spotting on solid slides using an arraying robotics. For target generation, RNA is isolated from cells, e.g. cultured at different growth conditions or from normal and diseased cells. In the next step, the targets are labelled with two different fluorescent dyes, usually Cy3 (green) and Cy5 (red), and reversed transcribed into cDNA. The two samples are pooled and hybridised to the probes on the microarray. Scanning of the slide after hybridisation is performed wit a laser scanner for imaging of Cy3- and Cy5-labelled cDNA probes (see fig. 1.4).

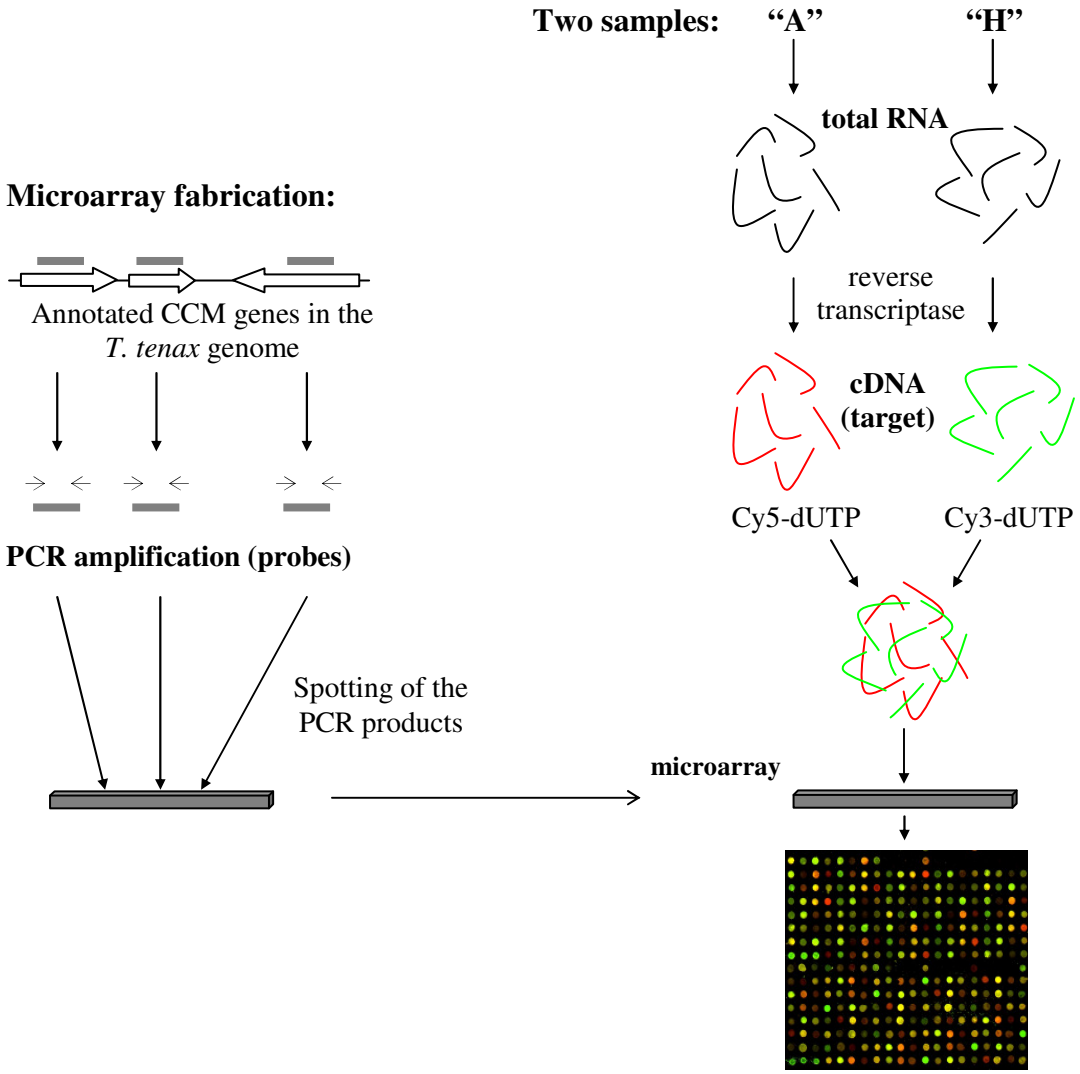


Fig. 1.4 **Scheme of DNA microarray fabrication and a two-sample hybridisation experiment.** (modified, Ehrenreich, 2006) **left:** Main steps of probe generation and microarray fabrication; **right**: Hybridisation procedure. Sample A from *T. tenax* grown autotrophically on CO_2 and sample H from *T. tenax* grown heterotrophically on glucose.

CCM regulatory proteins

Beside the investigation of transcriptional regulation using the *T. tenax* cDNA microarray, additional studies were performed in order to identify transcriptional regulatory proteins of *T. tenax*.

As previously mentioned, archaeal information processing pathways resemble eucaryal systems. The archaeal basal transcription machinery is similar to the core components of the eucaryal transcription apparatus (Huet *et al.*, 1983; Bell and Jackson, 1998; Bell *et al.*, 2001),

composed of a RNA polymerase (RNAP) and transcription initiation factors. Archaea are shown to possess a eucaryal RNAPII and homologs of the two general transcription factors TATA-box binding protein (TBP) and transcription initiation factor IIB (TFIIB) (Bell and Jackson, 1998; Bell *et al.*, 2001).

Compared to the current knowledge of the basal transcription apparatus and the steps of transcription initiation, little is known about the mechanisms of modulation of gene expression in Archaea.

Surprisingly, archaeal genome analyses (Kyrpides and Ouzouni, 1999; Aravind and Koonin, 1999) revealed the presence of several homologs of bacterial-type regulators. Therefore, transcriptional regulation in Archaea is accomplished by bacterial-type regulators that interact with an eucaryal-like transcription machinery.

Some transcriptional regulators have already been described in Archaea, e.g. the metal-dependent repressor 1 (MDR1) from *Archaeoglobus fulgidus* (Bell *et al.*, 1999) and a few archaeal specific regulators have already been identified, e.g. Phr, a regulator of heat shock response in *P. furiosus* (Vierke *et al*., 2002) or TrmB, which regulates expression of a trehalose/maltose transport operon in *Thermococcus litoralis* (Lee *et al.*, 2003).

However, recent genome analyses revealed that members of the bacterial-type Lrp (leucine-responsive regulator protein) family of transcriptional regulators (COG1522) are also widely distributed among the Archaea (Brinkman *et al.*, 2003). In Bacteria, Lrp represents a global regulator, which is involved in the transcriptional regulation of several genes, mainly involved in amino acid or nitrogen metabolism. Lrp can either induce (positive) or repress (negative gene regulation) transcription of specific genes and the regulator typically represses transcription of its own gene by binding to its own promoter region and therefore prevent RNAP binding.

Several archaeal homologs have already been characterised, e.g. LrpA from *P. furiosus* (Brinkmann *et al*., 2000; Dahlke and Thomm, 2002). LysM from *S. solfataricus* was shown to induce transcription of a lysine biosynthesis operon (*lysWKJK*; Brinkmann *et al*., 2002).

Seven members of the Lrp/AsnC family of transcription regulators were identified in the *T. tenax* genome and the present work will contribute to elucidate the function of these putative transcriptional regulators in *T. tenax*.

Investigations of the trehalose metabolism of T. tenax

The presence of trehalose in *T. tenax* has been reported by Martins *et al.* (1997). Further on, the reconstruction of the *T. tenax* trehalose metabolism revealed the presence of the so called OtsA/OtsB pathway (trehalose-6-phosphate synthase (TPS/OtsA)/ trehalose-6-phosphate phosphatase (TPP/OtsB) in *T. tenax* (Siebers *et al.,* 2004) that was so far only identified in Eucarya and Bacteria (Giaever *et al.*, 1988; Kaasen *et al.*, 1994).

The disaccharide trehalose

Trehalose is a nonreducing disaccharide composed of two α,α-1,1-glycosidic linked glucose molecules (see fig. 1.5) and is present in a great variety of organisms including Bacteria, Archaea, fungi, plants, invertebrates and mammals. Beside an initially supposed function as carbon and energy source (Elbein, 1974), trehalose is shown to play a crucial role in stress adaptation (compatible solute) in Bacteria and Eucarya. It protects organisms, their proteins and membranes against various stress factors, e.g. temperature (heat, cold), high osmolarity, oxidative stress or dehydration. It was demonstrated that trehalose is predominantly accumulated in Bacteria in response to high osmolarity, e.g. shown for *E. coli* (Strom and Kaasen, 1993), whereas in Eucarya, the disaccharide seems to be mainly involved in thermoadaptation, e.g. in yeast *Saccharomyces cerevisiae* (Hottiger *et al.*, 1987; De Virgilio *et al.*, 1994).

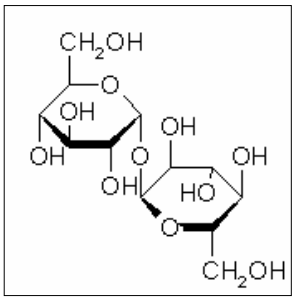


Fig. 1.5 **Structure of trehalose**

Beside in *T. tenax,* the presence of trehalose has also been reported in several members of the Archaea, e.g. *Pyrobaculum aerophilum*, *Thermoplasma acidophilum*, *Sulfolobus solfataricus*, *Acidianus ambivalens* and *Metallosphaera sedula* (Nicolaus *et al.*, 1988; Martins *et al.*, 1997). However the respective synthesis pathways are not studied in more detail and so far, the physiological function of the disaccharide in Archaea is still unknown. For thermophilic and hyperthermophilic Archaea trehalose is discussed as a thermoprotectant. In halophilic Archaea it is supposed to function as an osmoprotectant (Martins *et al.*, 1997), whereas a possible additional role as carbon and energy source cannot be excluded. Recently, 2-sulfotrehalose (sulfate at C-2) was identified as important compatible solute for osmoadaptation in haloalkaliphilic Archaea, e.g. *Natronococcus*- species (Desmarias *et al.*, 1997).

The reason for the widely distribution of trehalose in numerous different organisms consists in its physical and chemical properties, which include high hydrophilicity, high thermo- and pH-stability and the absence of internal hydrogen bonds.
The disaccharide maintains membrane integrity and protein stability under various stress conditions, e.g. heat, dehydration, high osmolarity or freezing (Crowe *et al.*, 1984 and 1992). Due to its characteristic properties, trehalose finds broad industrial application, e.g. in food or pharmaceutical industry. It has become a widely valued preservative (Argüelles, 2000).

Intracellular accumulation of trehalose
At least four different pathways for the biosynthesis of trehalose have been described (see fig. 1.6). The most common and best studied synthesis pathway known from Bacteria and Eucarya, involves the enzymes trehalose-6-phosphate synthase (TPS; OtsA in *E. coli*) and trehalos-6-phosphate phosphatase (TPP; OtsB in *E. coli*) (Kaasen *et al.*, 1994; Giaever *et al.*, 1988). TPS catalyses the transfer of glucose from UDP-glucose (UDPG) to glucose 6-phosphate (G6P) forming trehalose 6-phosphate (Tre6P) and UDP, subsequently TPP dephosphorylates Tre6P yielding trehalose (see fig 1.6).
Several mesophilic Bacteria, e.g. belonging to the genera *Arthrobacter, Brevibacterium* or *Micrococcus* as well as thermophilic Archaea (members of the *Sulfolobales*) produce trehalose from starch via the TreY-TreZ-pathway (Maruta *et al.*, 1996; Kobayashi *et al.*, 1996; Di Lernia *et al.*, 1998; Gueguen *et al.*, 2001; see fig 1.6).
A third pathway for trehalose synthesis catalysed by trehalose synthase (TreS) is described for bacterial species, e.g. *Thermus aquaticus* (Tsusaki *et al.*, 1997) and was recently discovered in the hyperacidophilic, thermophilic Archaeum *Picrophilus torridus* (Chen *et al.*, 2006). TreS converts the α-1,4-linkage of the disaccharide maltose into an α-1,1-linkage forming trehalose (see fig 1.6).
Recently, a fourth pathway was described for the marine Archaeum *Thermococcus litoralis*, which is characterised by a glycosyl-transferring trehalose synthase (TreT). The enzyme catalyses the reversible formation of trehalose from UDPG or ADPG and glucose (Qu *et al.*, 2004), but due to its gene organisation in an operon comprising genes coding for trehalose/maltose transport system, the enzyme is discussed a preferred trehalose degrading enzyme.

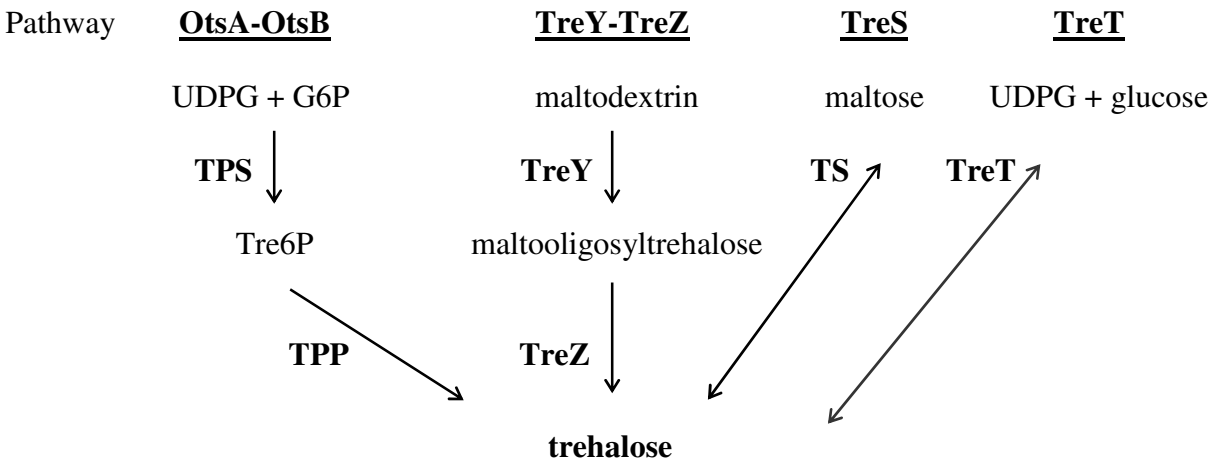


Fig. 1.6 **Metabolic pathways involved in the biosynthesis of trehalose.** TPS: trehalose-6-phosphate synthase (OtsA in *E. coli*), TPP: trehalos-6-phosphate phosphatase (OtsB in *E. coli*); TreY: maltooligsyl-trehalose synthase, TreZ: maltooligosyl-trehalose hydrolase; TS: trehalose synthase; TreT: glycosyl-transferring trehalose synthase

So far, only alternative pathways to common bacterial and eucaryal OtsA/OtsB pathway have been described in Archaea, e.g. the TreY-TreZ pathway for members of the *Sulfolobales* (Maruta *et al.*, 1996), the TreT described for *T. litoralis* (Qu *et al.*, 2004) or the TreS of *Picrophilus torridus* (Chen *et al.*, 2006).

However, in the genome of *T. tenax* one gene was identified, which codes for a trehalose-6-phosphate synthase/phosphatase, a fusion protein (TPSP; *tpsp* gene) comprising a N-terminal TPS and a C-terminal TPP domain. First functional analyses of the single TPS (Brenner, 2001) and the single TPP domain (Zaparty, 2003) revealed that the OtsA/OtsB pathway is active in *T. tenax* (Siebers *et al.*, 2004). The present work has been performed to further analyse and elucidate the enzymatic properties of the TPSP and of two additional gene products, which are organised in an operon with the tpsp gene.

In general, several pathways for the hydrolysis of trehalose are described in Bacteria and Eucarya, but the most common pathway is represented by trehalases. Some prokaryotes and eukaryotes, e.g. *Euglena gracilis* (Marechal and Belocopitow, 1972), catalyse the reversible phosphorolysis of trehalose to glucose 1-phosphate and glucose via the trehalose phosphorylase (TreP). Phosphotrehalase (TreC) converts trehalose 6-phosphate to glucose 6-phosphate and glucose, e.g. TreC from *E. coli* (Rimmele and Boos, 1994) and *Bacillus subtilis* (Helfert *et al.*, 1995). However, virtually nothing is known about trehalose degradation pathways in Archaea, with the exception of the TreT from *T. litoralis*.

Aims of the work

The present work will contribute to the further elucidation of the regulatory processes and networks of the central carbohydrate metabolism (CCM) of *T. tenax*, by using the DNA microarray technique.

The constructed microarray has been used to investigate transcriptional regulation of the CCM genes in response to heterotrophic growth on glucose compared to autotrophic growth on CO_2 in order to elucidate the regulation of the catabolic and anabolic carbon flux in *T. tenax*.

Furthermore, studies of the regulatory function of the putative transcriptional regulatory protein Lrp1, shall reveal information about a possible coordinative transcriptional regulation of the ED genes involved in the branched ED pathway of *T. tenax*.

In addition to the investigations of adaptation to different carbon sources, studies of the trehalose metabolism are part of this work, in order to enlighten how *T. tenax* adapts to other environmental factors, such as stress conditions.

2 MATERIALS AND METHODS

2.1 Chemicals, enzymes, kits and consumables

[α-^{32}P]-CTP	Hartman Analytics, Braunschweig
Acrylamide, N,N-methylenebisacrylamide	SERVA GmbH, Heidelberg
Alkaline phosphatase, calf intestinal (HC)	Promega GmbH, Mannheim/ Madison (USA)
Anti-digoxigenin-AP, Fab-fragments	Roche Diagnostics GmbH, Mannheim
Antibiotics	Sigma-Aldrich, Taufkirchen
Blocking reagent	Roche Diagnostics GmbH, Mannheim
Bradford Reagent	BioRad, München
CDP-Star (Tropix)	Roche Diagnostics GmbH, Mannheim
Cy5-/Cy3-dUTP	GE Healthcare
Betaine monohydrate	Sigma-Aldrich, Taufkirchen
Bovine serum albumin (BSA)	Sigma-Aldrich, Taufkirchen
Comassie-Brilliant-Blue R and G	SERVA GmbH, Heidelberg
Denhardt's solution	Sigma-Aldrich, Taufkirchen
Dialysis tubes	Medicell International, London (UK)
Diethyl pyrocarbonat (DEPC)	Sigma-Aldrich, Taufkirchen
DIG Oligonucleotide 3'-end Labeling Kit, DIG-ddUTP	Roche Diagnostics GmbH, Mannheim
DIG RNA-Labeling-Kit (SP6/T7)	Roche Diagnostics GmbH, Mannheim
DNA-ladder (1 kb), GeneRuler^{TM}, ready-to-use	Fermentas Life Sciences
DNA-Polymerase, *Thermus aquaticus*	Fermentas Life Sciences
DNA-Polymerase, *Pyrococcus furiosus*	peQLab Biotechnologie GmbH, Erlangen
DNase, on-column	Qiagen, Hilden
dNTP Mix	Fermentas Life Sciences
DNAzol Reagent	Invitrogen
Hexamer mix (NNNNNA, NNNNNT, NNNNNC and NNNNNG)	Sigma-Aldrich, Taufkirchen
HotStart*Taq* polymerase kit	Qiagen, Hilden
Hybri-Slip coverslip (22x22 mm)	Sigma-Aldrich
Isopropyl-ß-thiogalactopyranosid (IPTG)	Gerbu Handelsgesell., Gaiberg
Kieselgel 60 DC-Platten (10 x 20 cm)	Merck Biosciences, Darmstadt
Microcon YM-30 centrifugal filter units	Millipore
Rotilabo Microtest-plates (Polystyrene, V-profile)	Roth GmbH, Karlsruhe
Molecular mass standard, SDS-7 and SDS-6H	Sigma-Aldrich
Nytran N, nylon transfermembrane	Schleicher & Schuell, Dassel
Oligonucleotides for cloning and sequencing	Sigma ARK
Oligonucleotides for microarray construction	MWG Biotech
Polylysine-coated glass slides, Poly-Prep^{TM}	Sigma diagnostics
ProBlott membrane (PVDF)	Applied Biosystems
QIAGEN plasmid-kit, QIAquick Gel Extraction	

kit, QIAquick Nucleotide Removal kit, QIAquick PCR Purification kit	Qiagen, Hilden
Restriction endonucleases	Fermentas Life Sciences New England Biolabs, Frankfurt
Prestained Protein Molecular Weight Marker	Fermentas Life Sciences
RBS 50-decontamination-solution	Roth GmbH, Karlsruhe
Reverse transcriptase (M-MLV RT, RNase H minus)	Promega GmbH, Mannheim/ Madison (USA)
rNTP Mix	Roche Diagnostics GmbH, Mannheim
RNA ladder, high range	Fermentas Life Sciences
RNase A	Merck Biosciences, Darmstadt
RNase away	Roth GmbH, Karlsruhe
RNeasy Mini Kit	Qiagen, Hilden
Scintillation liquid	Rotiszint eco plus, ready-to-use, Roth GmbH, Karlsruhe
SDS	SERVA GmbH, Heidelberg
T4-DNA-ligase	Fermentas Life Sciences
T7-RNA-Polymerase	Fermentas Life Sciences
T7 *in vitro* transcription kit	Fermentas Life Sciences
TRIzol Reagent	Life Technologies, Karlsruhe
ULTRAhyb solution	Ambion
Whatman GB 004, 3MM	Schleicher & Schuell GmbH, Dassel
X-Ray developer	LX24, Kodak
X-Ray fixer	AL4, Kodak
Yeast t-RNA	Roche Diagnostics GmbH, Mannheim

Remaining chemicals unlisted above were purchased from Fluka and Riedel de Haen (Seelze), Gerbu (Gaiberg), Sigma-Aldrich (Taufkirchen), Roth GmbH (Karlsruhe), Difco Laboratories (Augsburg) and VWR International (Darmstadt) in analytical grade as well as organic solvents purchased from J.T. Baker B.V. Deventer (NL).

2.2 Instruments

Aqua bidest. water system	Seral Pro 90 CN, Elga-Seral, Ransbach-Baumbach
Agarose gel electrophoreses	Agagel Mini, Biometra, Göttingen; Power supply: Consort E143 (MS Laborgeräte)
Autoclave	Webeco Modell H, Webeco GmbH, Bad Schwartau

Autoradiography	BioMax ML Imaging films, BioMax cassette equipped with BioMax MS Intensifying screen, Kodak; BAS cassette 2025 equipped with imaging plate, Fuji Film
Benchtop heater	BT3, Grant Instruments, Cambridge, UK
Capillary cooler	Self-made by the fine Mechanics Dept., University of Duisburg-Essen
Cell disruption/homogenisation	French Press, SLM Aminco Instruments Inc., distributed by Sopra GmbH, Büttelborn; hand held glass-teflon homogenizer, B. Braun AG, Melsungen
Centrifuges	Bench centrifuges: Sigma 3K12, B. Braun AG, Melsungen; Hettich Universal centrifuge 32R; Biofuge® pico and Biofuge A, Heraeus Instruments; large centrifuge: Avanti J-25, Beckmann, München; Ultracentrifuge: L8-80, Beckman Coulter GmbH, Krefeld Eppendorf 5810R (Rotor A-4-62 with Microtiter plate carrier), Eppendorf, Hamburg
Chemiluminescence detector for gel documentation, including video copy processor:	ChemiDoc Gel Documentation System, BioRad Laboratories GmbH, München, P91W B/W thermal printer, Mitsubishi
Chromatographie	Columns and Ni-NTA agarose, Qiagen Hilden
Contamination monitor	Contamat FHT 111M LB 124, CA (USA), Berthold Technologies GmbH Bad Wildbad
Dispersing	Ultra Turrax T25, IKA, Staufen
Fermenter	Biostat® 100 L Fermenteranlage, B. Braun AG, Melsungen Heat exchanger: Integra T10000 460 / 60HZ 3P Lauda
Hybridisation Chamber	Corning CMT^{TM}
Hybridisation oven	OV3 Mini hybridisation oven and Compact Line OV4, Biometra, Göttingen

Incubators	Certomat H / Certomat R, B. Braun AG, Melsungen; Minitron Incubator Infors AG, Bottmingen, Basel, Switzerland
Microscopes	Olympus BH-2 RFCA and Olympus CHT, Olympus, Hamburg
Phosphor imager and data processing	Image Reader FLA 5000, V2.1, Fuji Film; AIDA software, Fuji Film
Photometer	Specord 200, Analytik Jena AG, Jena with WinASPECT Spectralanalysis-Software; BioPhotometer and Eppendorf 1101M, Eppendorf, Hamburg
Polyacrylamide gel electrophoresis (SDS-PAGE)	Mingel-Twin, Biometra, Göttingen; Power supply: Consort E835, MS Laborgeräte
Protein electrotransfer chamber	CarboGlass semidry chamber, Schleicher & Schuell
Robot for microarray printing	MicroGrid II spotter, BioRobotics
Scanning of the microarrays and image analyses	GenePix 4000a scanner including GenePix Pro Software (3.0), Axon instruments; GeneSpring software, Silicon genetics (kindly provided by Prof. Dr. J. Soppa)
Scintillation Counter	Liquid Scintillation Counter Wallac 1409, PerkinElmer, Wallac, Turku, Finnland
Temperature probe	P510 Temp, Dorstmann Electronic GmbH, Wertheim
Thermal cycler	iCycler, appropriate thin-wall PCR tubes, BioRad Laboratories GmbH, München; Uno II, Biometria, Göttingen
UV crosslinker	Stratalinker 1800, Stratagene, Leiden, NL
UV light	Konrad Benda N90, MW312 nm, Wiesloch
Vacuum centrifuge	SpeedVac Concentrator, Savant Farmingdale, UK
VIVASPIN columns	VIVASCIENCE, Sartorius group, Stonehouse, UK

2.3 Strains and culture conditions

Thermoproteus tenax Kra1 strain; DSMZ 2078 (Zillig *et al*., 1981)
Escherichia coli K12 DH5α strain; DSMZ 6897 (Hanahan, 1983)
Escherichia coli BL21(DE3); Novagen (Studier and Moffatt, 1986)
Escherichia coli Rosetta(DE3); Novagen
Escherichia coli BL21(DE3) pLysS; Novagen (Studier and Moffatt, 1986)
Escherichia coli BL21-CodonPlus(DE3) pRIL; Stratagene (Carstens and Waesche, 1999)
Sulfolobus solfataricus pyrEF mutant strain PH1-16, (Martusewitsch *et al.*, 2000; kindly provided by Dr. S.V. Albers, University of Groningen (NL))

Mass cultures of *Thermoproteus tenax* Kra 1 were grown at 86°C and pH 5.6 in an enamelled 100-l fermenter (B. Braun Biotech International, Melsungen) in a complex medium according to Brock *et al.* (1972; modified) containing (amount per litre): 1.3 g $(NH_4)2SO_4$, 0.28 g KH_2PO_4, 0.25 g $MgSO_4$ x $7H_2O$, 0.07 g $CaCl_2$ x $2H_2O$, 0.02 g $FeSO_4$ x $7H_2O$, 1.8 mg $MnCl_2$ x $4H_2O$, 4.5 mg $Na_2B_4O_7$ x $10H_2O$, 0.22 mg $ZnSO_4$ x $7H_2O$, 0.05 mg $CuCl_2$ x $2H_2O$, 0.03 mg Na_2MoO_4 x $2H_2O$, 0.03 mg $VOSO_4$ x $5H_2O$, 0.01 mg $CoSO_4$ x $7H_2O$, 1 mg resazurin. However, 5 g/l dispersed elemental sulphur (S^0) and 0.01 g/l yeast extract were added and anaerobic growth conditions were achieved by the addition of Na_2S x $7\text{-}9H_2O$. For heterotrophic growth, 2 g/l glucose was added. Cultures were continuously gassed with 80% H_2 / 20% CO_2 (v/v) under autotrophic growth conditions or with 80% H_2 / 20% N_2 (v/v) under heterotrophic growth at a flow rate of 1 l/min and stirred at 250 rpm. The fermenter was inoculated with 0.5-1 l of a preparatory culture (1 x 10^8 cells/ml) and cultivation was carried out over a period of about one week. Heterotrophic cultures reached a cell density of about 1 x 10^8 cells/ml, whereas autotrophic grown cultures reached up to 3-4 x 10^8 cells/ml. The cells were quickly cooled down to 4°C by the passage through a capillary cooler and harvested in early exponential (1-2 x 10^7 cells/ml), exponential (6-7 x 10^7 cells/ml) and stationary growth phase (1 x 10^8 cells/ml) by centrifugation (10,000 x *g*, 20 min, 4°C) and finally the cells were stored at -80°C.

Cultivation of the *pyrEF* mutant strain *S. solfataricus* (PH1-16) was carried out in the laboratory of Dr. S.V. Albers (Groningen University, The Netherlands) in a medium according to Brock *et al.* (1972) containing (amount per litre): 2.5 g $(NH_4)_2SO_4$, 3.1 g KH_2PO_4, 203.3 mg, $MgCl_2$ x $6H_2O$, 70.8 mg $Ca(NO_3)_2$ x $4H_2O$, 2 mg $FeSO_4$ x $7H_2O$, 1.8 mg $MnCl_2$ x $4H_2O$, 4.5 mg $Na_2B_4O_7$ x $2H_2O$, 0.22 mg $ZnSO_4$ x $7H_2O$, 0.06 mg $CuCl_2$ x $2H_2O$, 0.03 mg Na_2MoO_4 x $2H_2O$, 0.03 mg $VOSO_4$ x $2H_2O$ and 0.01 mg $CoCl_2$ x $6H_2O$. The

medium contained 0.1 % trypton and cells were grown either with or without uracil (10 µg/ml). Optical densities of liquid cultures were monitored at 600 nm (OD_{600}). Cells were grown aerobically in a rotary shaker at 80°C and pH 3.0.

The aerobic cultivation of *E. coli* was carried in 3 – 400 ml batch cultures in reaction tubes or Erlenmeyer flasks at 37°C in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, 1% NaCl (w/v), pH 7) or on solid medium plates (LB medium containing 1.5% (w/v) agar-agar). An optimal oxygen supply of the smaller liquid cultures (3 - 400 ml) was given by strong shaking (220 rpm). The aeration of mass cultures (volumes up to 15 litres) was achieved by gassing compressed air through a bacterial tight filter with a flow rate of 50 l/min. Antibiotics were added according to the plasmid-encoded antibiotic resistance (see tab. 1) in the following concentrations: ampicillin 100 µg/ml, kanamycin 50 µg/ml and chloramphenicol 34 µg/ml.
The *E. coli* strain K12 DH5α was used for cloning, storage and preparation of plasmid-DNA. The strains *E. coli* BL21(DE3), BL21(DE3) pLysS, BL21-CodonPlus(DE3)-RIL and Rosetta (DE3) pRIL and the pET Vektor System (Novagen) (see tab. 1) were used for the heterologous expression of recombinant *T. tenax* proteins.
Liquid LB medium containing the appropriate antibiotic was inoculated with a preculture (1 % (v/v)) and growth was monitored spectrophotometrically at 578 nm. Protein expression was induced at OD_{578} = 0.6–0.8 by the addition of 1 mM IPTG and incubation was continued for 3 – 4 hours. Afterwards, cells were chilled on ice and harvested by centrifugation (6,000 x *g*, 15 min, 4°C) and finally stored at -80°C.

2.4 Plasmids and constructed recombinant vectors

Tab. 2.1 **Plasmids and their application.**

Vector	Resistance	Application	Source of supply
pET15b	Amp^r	Heterologous expression of *T. tenax* proteins in *E. coli*	Novagen, Merck Biosciences
pET24a	Kan^r	Heterologous expression of *T. tenax* proteins in *E. coli*	Novagen, Merck Biosciences
pMZ1	Amp^r	Cloning of *T. tenax msc* for	provided by Dr.S.V.Albers,

		expression in *S. solfataricus* (Albers *et al.*, 2006) contains C-terminal tandem(strep-his)-tag	University Groningen (NL)
SSV1		*S. solfataricus* shuttle vector (Jonuscheit *et al.*, 2003; Albers *et al.*, 2006)	Dr. S.V.Albers, University of Groningen (NL)
pSVA80		Expression-plasmid containing *T. tenax msc*	Dr. S.V.Albers, University of Groningen (NL)
pLysS	Cam^r	Heterologous expression of T7 lysozyme in *E. coli*	Novagen, Merck Biosciences
pRIL	Cam^r	Expression of rare tRNA genes (*argU*, *ileY*, *leuW*)	Stratagene, La Jolla (USA)
p*rpoS*	Amp^r	*In vitro* transcription (Zaigler *et al.*, 2003), pSK plasmid (pPCR-Script; PCR-ScriptTM Amp cloning Kit, Stratagene)	Provided by Dr. A. Zaigler & Prof. J. Soppa, University of Frankfurt
pTrCH6	Amp^r	Expression of mechanosensitive channel (membrane protein), contains C-terminal his-tag	Prof. I. Booth, Scotland

E. coli Rosetta(DE3) contains a plasmid encoding *argU, argW, glyT, IleX, leuW, proL.* Therefore, the host allows expression of genes encoding tRNAs for the rare argenine (AGA, AGG, CGA), glycine (GGA), isoleucine (AUA), leucine (CUA), and proline (CCC) codons.

Tab. 2.2 **Constructed recombinant vectors.** The primer sets and their sequences are given. The restriction recognition sites (framed) and the oligo-attached histidine-tag (6x his; underlined) are indicated. For expression in *E. coli,* amplification of the *tpsp* gene was performed via Synf-*Nde*I-2 or *tpsp*24a-his-*Nde*I-F and thereby the start codon was changed from GTG (valine) to ATG (methionine).

Plasmid + insert	Primer set	sequence (5'→ 3')	T_m
pET15b+*hp5*	*hp5-Nco*I –f	GCTAGCCCATGGTCAAATATCTG	64°C
	*hp5-Bam*HI-rev	ATCTGGGATCCTAAATTATATGTTC	68°C
pET15b + *lrp1*	*lrp1-Nco*I–fII	AGCCCATGGACGAGATAGACAGG	64°C
	*lrp1-Bam*HI-revII	AAGTGGGGATCCTGAAGCGCT	55°C
pET24a + *lrp1* his (Lrp1 His)	*lrp1*-his-*Nde*I-f	AAAAACATATGCATCACCATCACCATCACGTGGACGAGATAGACAGGAAGCTTA	55°C
	*lrp1-Bam*HI-revII	AAGCGCTTATAAGCGGATCCTCCTA	55°C

pET24a + *tpsp*	Synf-*Nde*I-2	TTCCGTGGGAGGACATATGCG	64°C
	RevTPSP-*EcoR*I-2	CGCCAGCGGCGAATTCTAGAGACAGGGG	67°C
pET24a + *tpsp*his	*tpsp* 24a-his-*Nde*I-F		50°C
(TPSP His)	AAAAACATATGCATCACCATCACCATCACATGCGCCTCATAGTGGTCTCC		
	RevTPSP-*EcoR*I-2	(see above)	
pET24a + *gt*	*gt-Nde*I-f II	GCCATATGCGCCGTCGTGATC	66°C
	g*t-Eco*RI-revII	CCACCGGAATTCCTTTTTACG	58°C
pET24a + *gt* his	*gt*-his-*Nde*I-f		52°C
(GT His)	AAAAACATATGCATCACCATCACCATCACATGAACGTAGCTGTAGTGGCGCC		
	g*t-Eco*RI-revII	(see above)	
pET24a + *msc*	*hp-Nde*I-f	ATATTCGCGCGGCGGGCCCCATATGGGACT	68°C
	*hp-EcoR*I-rev	CCAACGGGAATTCTGCGGCGC	65°C
pET24a + *msc* his	*hp-Nde*I-f	(see above)	
(MscTTX C-his1)	*msc*-his-C-*Xho*I-rev	GCGGCGCCACTACAGCCTCGAGGCAT	69°C
pET15b + *msc*his	*hp-Nde*I-f	(see above)	
(MscTTX N-his2)	*msc*-his-N-*Xho*I-rev	GCGGCGCCACTACAGCTCGAGTCAT	64°C
pTrcH6 + *msc* his	*msc*-his-pTrC-*Nco*I-f III		
(MscTTX C-his3)		CGGCGGGCCCACCATGGGACTCTTGGAATA	67°C
	msc-his-pTrC-*Xho*I-rev III		
		GGCGCCACTACAGCCTCGAGTCTTTTGA	68°C
pMZ1 + *msc*	*msc-Bsp*HI-f	GGCGGGCTCATGATGGGACTCTTGG	67°C
	*msc-BamH*I-rev	CTACAGCTGGATCCTCTTTTGAGGAGC	60°C

Existing constructs:

pET24a + *syn-2*	*syn2-Nde*I-f	TTCCGTGGGAGGACATATGCG	64°C
(Brenner, 2001)	*syn-Eco*RI-rev	GCCGAATTCGCCGAGATTAGGGA	68°C
pET24a + *phos*	*phos-Nde*I-f	GAGAAGGCCCTCAGACATATGGA	68°C
(Zaparty, 2003)	*phos-Eco*RI-rev-a	GGTTGAATTCTTAGCCCGCGG	66°C

2.5 Biomolecular techniques: Working with DNA

2.5.1 Preparation of genomic DNA from *T. tenax*

For the preparation of genomic *T. tenax* DNA 0.1 g cells were homogenised and lysed in 2 ml DNazol reagent. The preparation was carried out in accordance with the manufacturer's instructions, with slight modifications. The method is based on the use of a guanidinium-detergent lysing solution that hydrolyses RNA and promotes the selective precipitation of DNA from the cell lysate (Chomczynski *et al*., 1993; Mackey *et al*., 1996). The cells were homogenised by using a hand-held glass-teflon homogeniser. After centrifugation (10,000 x *g*, 10 min, RT) the supernatant was decanted, transferred in a fresh tube and 1 ml ethanol (100%) was added. After gently inverted the tube for a few times the sample was incubated at RT for 3 min. The precipitated DNA was sedimented by centrifugation (10,000 x *g*, 10 min, RT). The supernatant was decanted and the DNA was washed twice with 1 ml ethanol (70%) and then centrifuged again. The remaining ethanol was completely removed under vacuum (speed vac) and the pelleted DNA was then dissolved in 100 µl aqua bidest. and incubated at room temperature for 10 min. To better dissolve the DNA, 1 vol 16 mM NaOH was added. Aliquots of the DNA were stored at -20°C and -80°C.
The DNA preparations were quantified photometrically at $\lambda = 260$ nm. The quality of purified DNA was checked by restriction enzyme digestion, subsequent agarose gel electrophoresis and the A_{260}/A_{280} ratio (see 2.5.4-5 and 2.5.8).

2.5.2 Preparation of plasmid DNA from *E. coli*

Preparation of plasmid DNA by alkaline lysis according to Birnboim and Doly (1979; modified)
Plasmid-DNA isolated by this alkaline lysis method was used for restriction analyses and cloning procedures. 2 ml of an overnight culture was centrifuged (12,000 x *g*, 5 min RT) and the cell pellet was resuspended in 200 µl buffer 1 (50 mM Tris, 10 mM EDTA, pH 8, 100 µg/ml RNase A) followed by the addition of 300 µl buffer 2 (200 mM NaOH, 1% (w/v) SDS) leading to cell lysis during incubation at RT for 5 min. Genomic DNA was precipitated by adding 300 µl chilled buffer 3 (3 M K-acetate, pH 4.8) and adjacent incubation on ice for 20

min. After centrifugation (20,000 x *g*, 15 min, 4°C) to remove the genomic DNA, the plasmid DNA was precipitated by the addition of 0.7 vol isopropanol and incubated at RT for 10 min. To recover the precipitated plasmid DNA, the sample was centrifuged (20,000 x *g*, 15 min, 4°C) the supernatant discarded and the plasmid-DNA pellet was rinsed with a diluted ethanol solution (1.0 ml 70% (v/v) ethanol). The pellet was completely dried under vacuum (speed vac) and finally resuspended in 50 µl aqua bidest.

Plasmid preparations with the QIAGEN plasmid kit

Plasmid-DNA used for quantitative and qualitative analysis was prepared with the QIAfilter Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions.

Rapid boiling-preparation of plasmid-DNA

This simple method for the rapid preparation of plasmid-DNA was used to perform a fast screening of recombinant *E. coli* clones. Single colonies of recombinant *E. coli* were picked with a sterilised pipette tip and a portion of the cells were streaked on an LB agar plate. The remainder was dissolved in 50 µl 10mM Tris/HCL, pH 7, incubated at 94°C for 5 min and finally centrifuged (14,000 x *g*, 1 min, RT). 5 µl of the resultant supernatant was used as template for PCR analyses in a 25 µl reaction volume.

2.5.3 DNA precipitation

DNA preparations were concentrated by ethanol or isopropanol precipitation (Sambrook *et al*., 1989). Hereunto, 2 volumes ethanol in presence of inorganic salt (1.5 M NH_4-acetate) were added to the sample and stored for at least 30 min at -20°C. Alternatively, 0.7-1 volume isopropanol can be used. After centrifugation (20,000 x *g*, 15 min, 4°C) and removal of the supernatant, the DNA pellet was rinsed with chilled 70% (v/v) ethanol and again centrifuged, the supernatant discarded and the DNA pellet dried under vacuum (speed vac). Finally, an adequate volume of aqua bidest. was added.

2.5.4 Quantitative and qualitative analysis of DNA

The concentration of the DNA preparations was determined photometrically by measuring the absorption of the sample at λ = 260 nm. An absorption of 1.0 (OD_{260} =1) corresponds to 50 µg of dsDNA/ml or 40 µg of ssDNA/ml (Sambrook *et al*., 1989). The purity of DNA was determined at 260 nm and 280 nm wavelengths.
Sufficient pure DNA preparations showed a ratio of absorbance (A_{260}/A_{280}) of > 1.8. Ratios less than 1.8 indicated that the preparation was contaminated, either with protein or phenol.

2.5.5 Agarose gel electrophoresis of DNA

The electrophoretic separation of DNA molecules by agarose gel electrophoresis (Sambrook *et al*., 1989) was used to determine the size and the amount of DNA molecules (genomic DNA, plasmid-DNA, PCR-products), to control progression of restriction enzyme reactions and to extract DNA fragments for cloning or sequencing reactions (see 2.5.6.1).
Depending on the size of the DNA fragments, agarose gels with 0.8% to 1.5% (w/v) in TAE-buffer (40 mM Tris-acetate, 1 mM EDTA) were used for electrophoresis. The dye ethidium bromide (EtBr) was added to the gel in a concentration of 0.5 µg/ml. EtBr intercalates between the bases of DNA and RNA and emits fluorescence when excited by UV light.
The DNA samples were mixed with loading dye (6x: 0.2% bromophenol blue, 0.2% xylene cyanol FF, 60% (v/v) glycerol, 60mM EDTA) and applied into the sample wells. 5 µl of a DNA marker containing a mixture of DNA fragments of known size and known amount (GeneRuler™, 1kb DNA ladder) was also applied onto the gel.
Electrophoresis was performed at 60–100 V depending on the gel size, in TAE-buffer at RT. After the electrophoresis run, the DNA was visualised by exposing the gel to UV light using a ChemiDoc-gel documentation system.
The sizes and concentrations of the separated DNA fragments were determined by comparison of their relative positions to those of the DNA strands of the DNA ladder.

2.5.6 Purification of DNA fragments

For the extraction and purification of DNA fragments from agarose gels or directly from the PCR assay, the QIAquick gel extraction kit and the QIAquick PCR purification kit, respectively, were used according to the instructions of the manufacturer (Qiagen).

2.5.7 Polymerase chain reaction (PCR)

The PCR technique enables the *in vitro* exponential enzymatic amplification of a specific nucleotide sequence in approximately two hours (Mullis *et al*., 1986; Saiki *et al*., 1988).
Two sequence-specific oligonucletide primers hybridise to the 5'–end of the coding and the non-coding strand, respectively, and flank the sequence region, that has to be amplified, and thus represent the starting points of elongation.
The elongation of the primers is catalysed by heat-stable DNA polymerases, e.g. the so-called *Taq*-polymerase of *Thermus aquaticus* or the *Pfu*-polymerase of *Pyrococcus furiosus*. The latter possesses an additional 3'-5' exonuclease activity ("proofreading-activity").
A standard PCR reaction includes the following main steps:

Denaturation: The dsDNA is denatured into ssDNA (template) at 94°C for 2 min;

Primer annealing: Hybridisation of the oligonucleotide-primers to their complementary DNA sequence;

Primer extension: DNA polymerase catalyses the elongation of the primers in 5'-3' direction and thus synthesises the polymerisation to dsDNA;

Final extension: Achieving complete synthesis of PCR products.

Cycling (25-30 x) of denaturation, primer annealing and elongation results in multiple copies of the target sequence.
Each primer possesses a specific annealing temperature, which depends on the length and the base composition of the oligonucleotide. The approximate melting temperature (T_m) for primers shorter than 20 nucleotides was calculated using the following formula (Thein & Wallace, 1986):

$$2 \times (n_A + n_T) + 4 \times (n_G + n_C) = T_m\,(°C)$$

2.5.7.1 Amplification of genomic DNA and plasmid DNA

The PCR amplifications were performed with 50-100 ng template DNA (genomic or plasmid-DNA), 1 µM of each primer (forward and reverse), 1.5 mM $MgCl_2$ and 200 µM dNTPs in 25–100 µl reaction mixture. The used reaction buffer depended on the employed DNA polymerase (*Taq*- or *Pfu* polymerase) and in general 1 U of the enzyme was used per 25 µl reaction mixture. The PCR reaction was performed using a thermal cycler.

2.5.7.2 PCR mutagenesis

For this PCR amplification, mutagenic primer sets were used to introduce restriction sites at the beginning and the end of specific DNA fragments. Subsequently, this modified DNA was ligated to accordant vectors (see tab. 2.2). To minimise the error rate, the *Pfu* DNA polymerase with additional proofreading activity was used.

2.5.8 Enzymatic modification of DNA

2.5.8.1 Restriction of DNA

The restriction of genomic DNA, plasmid-DNA and PCR products was carried out with the respective restriction endonucleases in accordant buffer following the instructions of the manufacturer. 2-3 U enzyme / µg DNA were used and the samples were incubated for 1-2 h at the recommended temperature.

2.5.8.2 5'-dephosphorylation of linearised vector-DNA

In order to avoid self-ligation of restricted vector DNA during the ligation reaction (see 2.5.8.3), the 5'-end phosphate groups were eliminated by calf intestinal alkaline phosphatase (CIP) treatment. 0.05 U of CIP/pmol DNA were added to the restriction reaction and incubated at 37°C for 30 min. The sample was then electrophoretically separated in an agarose gel (see 2.5.5) and purified (see 2.5.6).

2.5.8.3 Ligation

Ligation of restricted DNA fragments (inserts) into vector DNA is carried out by the T4 DNA ligase (Pan *et al*., 1994; Bankier *et al*., 1987). The enzyme catalyses the ATP-dependent formation of a phosphodiester linkage between the 5'-phosphoryl group and adjacent 3'-hydroxyl group of duplex DNA in a blunt-ended or cohesive-ended configuration.
Equimolar amounts of restricted, dephosphorylated plasmid DNA and insert were used at a ratio of 1:3 in a volume of 8 µl and incubated at 45°C for 5 min to break possible secondary structures. 1 µl of 10 x reaction buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8) and 1 µl T4 DNA ligase (1 weiss-unit/µl) were added to the reaction mixture yielding a final volume of 10 µl.
The ligation reaction was carried out overnight at 4°C. Subsequently, T4 DNA ligase was inactivated by incubation at 70°C for 10 minutes. The recombinant vector molecules were stored at -20°C or immediately used for transformation.

2.5.9 Transformation

2.5.9.1 Preparation of competent *E. coli* cells

Competent cells of *E. coli* (strains see 2.3) were chemically prepared by using rubidium chloride and calcium chloride (Promega Technical Manual, 1994). Therefore, 20 ml LB medium was inoculated with 0.2 ml from an overnight culture of *E. coli* and incubated at 37°C in a rotary shaker until OD578 0.3–0.5 was reached. The culture was then centrifuged (2,300 x *g*, 10 min, 4°C). The cell pellet was gently resuspended in 10 ml chilled solution A (10 mM MOPS, 10 mM RbCl, pH 7.0), centrifuged (2,300 x *g*, 10 min, 4°C), again gently resuspended in 10 ml chilled solution B (100 mM MOPS, 50 mM $CaCl_2$, 10 mM RbCl, pH 6.5) and incubated for 30 min on ice. After the final centrifugation (700 x *g*, 15 min, 4°C), the pelleted cells were resuspended in 2 ml chilled solution B and either were directly used for transformation (see 2.5.9.2) or were stored in presence of 15% (v/v) glycerol at -80°C for two weeks.

2.5.9.2 Transformation of the competent *E. coli* cells

Plasmid DNA was gently mixed with 200 µl of competent *E. coli* cells, then incubated on ice for 1 hour. Transformation was achieved by a heat shock at 42°C for 45 sec and subsequent storage on ice for 5 min. 500 µl of LB medium was added and incubated at 37°C for about 1 h in a rotary shaker. 100 µl of transformed cells were plated on LB agar plates containing the respective antibiotics. The remaining 600 µl cell suspension were centrifuged, pelleted cells were resuspended in about 100 µl LB-medium and plated.
After incubation of LB agar plates containing the respective antibiotics at 37°C overnight, colonies were screened for positive clones carrying the recombinant plasmid using boiling-preparing of plasmid DNA (see 2.5.2.3) followed by PCR or restriction digestion (see 2.5.8.1) of isolated plasmid DNA (see 2.5.2.1 and 2.5.2.2).

2.5.10 Sequencing

Automated DNA sequencing (Sanger *et al*., 1977) was performed at Seqlab Company (Göttingen), AGOWA (Berlin) and in the DNA Sequencing Service at the Medical Faculty, University Clinic of Essen. The following primer sets were used for plasmid sequencing and for internal sequencing of recombinant plasmids and PCR products respective sequence-specific primers were used:

Tab. 2.3 **Vector primer for dideoxy sequencing reaction.**

Primer	Sequence (5'-3')	T_m
pET-f	GGATAACAATTCCCCTCTAG	55°C
pET-rev	GCTCAGCGGTGGCAGCAGCC	55°C
pMZ1-f	AACAAAACGTCTTTTACGGAAATAT	52°C
pMZ1-rev	CGGCAATCTAATGAAAATGAGATTA	53°C
Internal sequencing of the *tpsp* gene:		
Phos-kon-rev	CGGGGACATTAGAGGCGA	58°C
Syn-rev	GCCGACTTCGCCGAGATTAGGGA	68°C
Phos-f	GAGAAGGCCCTCAGACATATGGA	66°C

DNA sequencing in course of the *T. tenax* genome sequencing project was performed in collaboration with Dr. H.-P. Klenk (e.gene, Feldafing, Germany) and Prof. Dr. S.C. Schuster (MPI für Entwicklungsbiologie, Tübingen, Germany / Pennsylvania State University, USA). Annotation of the genome was performed at the University of Duisburg-Essen, Department of Microbiology.

2.5.11 Computer analysis of nucleotide and amino acid sequences, and additionally used databases.

Software	Reference	Application
CHROMAS	McCarthy, unpublished	Visualisation of sequence chromatograms
FASTA	Pearson & Lipman, 1988	Formatting of nucleotide- and amino acid Sequences for submission to databases
BLAST	www.ncbi.nlm.nih.gov/BLAST/ Altschul *et al*., 1990; Altschul *et al*., 1997	Calculating DNA and protein sequence similarity and homology searches
SOSUI	http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html	Prediction of transmembrane helices Nagoya University (Japan)
TMHMM	www.cbs.dtu.dk/services/TMHMM-2.0	Prediction of transmembrane helices CBS (Denmark)
PredictProtein, APSSP	http://cubic.bioc.columbia.edu/predictprotein/ http://imtech.res.in/raghava/apssp/	Secondary structure prediction
COG database	www.ncbi.nlm.nih.gov/COG/	Clusters of orthologous groups
Clustal W 1.83	Thompson *et al*., 1994	DNA and protein sequence alignments
GeneDoc	Nicholas & Nicholas, 1997	Editing of CLUSTAL files (*.aln)
Oligo 3.4	Rychlik & Rhoads, 1989	Oligonucleotide design for cloning
PrimeArray0.82	Raddatz *et al*., 2001	Oligonucleotide design for microarray construction; kindly provided by Prof. C. Dehio, University of Basel
MAGPIE	Gaasterland & Sensen, 1996	Initial *T. tenax* genome annotations
GenDB	Meyer *et al*., 2003 www.gendb.genetik.uni-bielefeld.de	*T. tenax* genome annotations
IMG 2.0	Markowitz *et al*., 2006 www.img.jgi.doe.gov/cgi-bin/pub/main.cgi	Comparative genome analyses
UCSC Archaeal	Schneider *et al*., 2005	Comparative genome analyses

genome browser	www.archaea.ucsc.edu/	
LBMGE Genomics ToolBox, Paris-Sud	www-archbac.u-psud.fr/genomics /GenomicsToolBox.html	Comparative genome analyses, homology searches
BRENDA 7.1	Schomburg *et al*., 2000 www.brenda.uni-koeln.de	Enzyme database; comprehensive enzyme information, e.g. reaction, specifity, preparation, structure, stability
ExPasy proteomics Server	Gasteiger *et al*., 2003 www.expasy.org	Proteomics server: Analyses of protein sequences, structures and 2-D PAGE
String-search tool for the Retrieval of interacting proteins	www.string.embl.de	Genomic context analyses
KEGG Kyoto Encyclopaedia of genes and genomes	www.genome.jp/kegg	Pathway maps

2.5.11 Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assays (gel shift assays) represent a technique to evaluate DNA-binding properties of a protein and thus to study gene regulation. The principle of an EMSA is based upon the fact that DNA-protein complexes migrate more slowly through a non-denaturing polyacrylamide gel than free DNA.

2.5.11.1 Generation and 3'-end-labelling of DNA probes with digoxigenin

The probes were amplified employing *Pfu* polymerase (100 µl reaction volume), purified after gel electrophoresis and quantified photometrically (see 2.5.4-2.5.7).

Tab. 2.4 **Oligonucleotides for amplification of the promoter spanning regions.** PCR products of the respective promoter spanning regions (200 bp up- and downstream of the putative promoter) contain putative DNA-binding sites.

Name	Sequence (5'-3')	T_m	Length of probe
Pro-Lrp1-f	CCATGACGAAGGCGAGGACTT	65°C	
Pro-Lrp1-rev	TATATCAAGGACAGCGCCGGA	70°C	410 bp
Pro-ED-f	TCAGCTCCATCTTCTCTTGCA	65°C	
Pro-ED-rev	ACCACTCGTAGAAAGCCGCCG	70°C	467 bp
Pro-HP5-f	CCTCGGGAAGTCCAGCTCGCC	70°C	
Pro-HP5-rev	CGGCGCGATTGATATGAGAGA	69°C	412 bp

Labelling was carried out using the DIG oligonucleotide 3'-end labelling kit (Roche diagnostics) according to the supplier's instructions: 100 pmol of the probes were mixed with 4 µl reaction buffer (5x: 1 M potassium cacodylate, 1.25 mg/ml BSA, 0.125 M Tris/HCL, pH 6.6), 4 µl $CoCl_2$ solution (25 mM), 1 µl DIG-ddUTP (1 mM) and 1 µl terminal transferase (50 U/µl) in a total volume of 20 µl. The samples were incubated for 15-30 min at 37°C and afterwards chilled on ice. The reaction was stopped by the addition of 2 µl of a glycogen/EDTA solution and the DIG-labelled probes were precipitated by adding 0.1 M LiCl, 3 vol of ethanol and incubation at -20°C for 20 min. After centrifugation (12,000 x *g*, 20 min, 4°C) the supernatant was discarded and the pellet was washed with 70% ethanol, dried under vacuum (speed vac) and 20 µl aqua bidest. were added. The labelling efficiency was checked by dot blot analysis and for this 4 µl of different dilutions (10 ng to 0.1 pg) of the labelled probes were applied punctually to a nylon membrane (Nytran). After UV-crosslinking (λ = 254 nm, 3 min) the detection was carried out as described under 2.5.12. The probes were stored at -20°C.

2.5.11.2 Incubation assays, electrophoretic separation and immobilisation of DNA-protein complexes

The three PCR amplified and 3'-end DIG labelled DNA fragments, which contained putative DNA-binding sites, were incubated with two recombinant putative DNA-binding proteins of

T. tenax (Lrp1, leucine–responsive regulatory protein; HP5, hypothetical protein 5; preparation see 2.8.3.4). A standard incubation assay (15 µl volume) was composed of: 75 – 100 ng DIG-labelled probe and 3 µg recombinant protein in TEK buffer (10 mM Tris/HCl, pH 8 at RT, 10 mM KCl, 1 mM EDTA, 10 mM β-Me).

Competition experiments were performed, with the addition of 2 µg of salmon sperm DNA to the reaction mixture, previously fragmented by 5 minutes of UV treatment. The non-specific salmon DNA acted as competitor DNA, ensuring only specific DNA-protein interactions occurred.

The reaction mixture was incubated for 15 min at RT or 37°C, respectively, then mixed with 5 µl pre-heated (37°C) loading buffer (15 % (w/v) Ficoll 400, 0.005% (w/v) bromophenol blue in TEK buffer) and directly applied to a freshly prepared 4% native TBE- polyacrylamide gel (Sambrook *et al.*, 1989). The native PAGE run was performed in 0.5 x TBE electrophoresis buffer. The gels were pre-run for 30 min at 10 mA. The primary reasons for pre-running the gels were to remove traces of ammonium persulfate (APS), which was used to polymerise the gels, to equilibrate the glycerol, which was partly added as stabilizing factor to the gels, and to ensure a constant gel temperature due to a constant voltage. After loading the samples onto the gel, the electrophoretic dead time required for the samples to enter the gel matrix was minimised by running the gel at 15 mA. Subsequently, the run was continued at 10 mA for 60 min, followed by the transfer to positively-charged nylon membranes (Nytran). Prior to transfer the membranes were equilibrated in 0.5 x TBE buffer for 5 min. Blotting was carried out for 1 h 15 min at RT (see fig. 2.1), then the membrane was briefly washed in 10 x SSC buffer and the DNA was crosslinked for 3 min at $\lambda = 254$ nm.

Immunological detection of the DIG-labelled DNA was performed by using an alkaline phosphatase-conjugated anti-DIG antibody and an alkaline phosphatase substrate (CDP-Star; see 2.5.12).

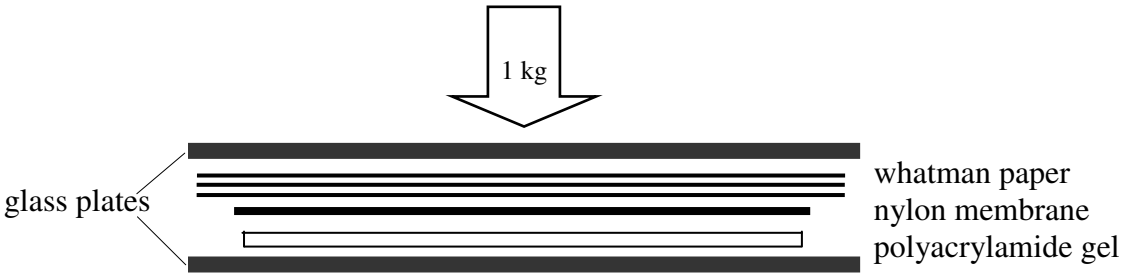


Fig. 2.1 **Blot assembly for transfer of DNA and DNA-protein complexes to a positively-charged membrane.**

2.5.12 Immunological detection of the DNA-protein complexes

The DNA-protein complexes were identified with an alkaline phosphatase-conjugated anti-DIG antibody by a chemiluminescence reaction. Detection of phosphatase activity was carried out by the alkaline phosphatase substrate CDP-Star (Tropix; Boehringer Mannheim, Manual 2001). The blot was equilibrated for 5 min in buffer 1 (0.1 M maleic acid, 3 M sodium chloride, pH 8) at RT, then 2 % (w/v) blocking reagent (Roche diagnostics) were dissolved in the buffer (buffer 2) and incubation continued for 1 h at RT on a rocking platform. Anti-digoxigenin-AP (Roche Diagnostics) with a final dilution of 1:20,000 was added to buffer 2 and incubated for 30 min at RT. Unbound antibody was washed off using buffer 1 (3 x 15 min) and finally the blot was equilibrated in the detection buffer (buffer 3: 0.1 M sodium chloride, 0.1 M Tris/HCl, pH 9.5) for 5 min at RT.
For the chemiluminscence reaction the membrane was placed in transparent films and the upside was wetted with the dye CDP Star. The chemiluminescence signals were detected using X-ray film (Kodak) or with the ChemiDoc Gel Documentation System (Bio-Rad) with an exposure time lasting from 10 sec to 30 min.

2.6 Biomolecular techniques: Working with RNA

2.6.1 Treatment of solutions, glassware and equipment

Working with RNA requires special measures to create and maintain a RNase-free environment. All applied buffers and solutions were mixed with 0.1% (v/v) diethyl pyrocarbonate (DEPC), which modifies histidyl-residues in proteins, e.g. RNases leading to inactivation. The treated solutions were incubated overnight at RT, then autoclaved to remove traces of DEPC. Glassware was heat sterilised by incubation at 210°C for at least 3 hours before use. Non-disposable plasticware was treated with 3% H_2O_2 and RNase Away (Roth). In order to keep solutions, reagents, and consumables, such as pipette tips, RNase-free, they were separated and used only for RNA work.

2.6.2 Isolation of total RNA from *T. tenax*

Total RNA was prepared from autotrophically and heterotrophically grown *T. tenax* cells harvested at different growth phases (early exponential: 1-2 x 10^7 cells/ml, exponential: 5-6 x 10^7 cells/ml and early stationary growth phase: 1 x 10^8 cells/ml) by using TRIzol reagent and RNeasy Mini Kit according to the instructions of the manufacturers with slight modifications. TRIzol reagent, a monophasic solution of phenol and guanidinium isothiocyanate, was used followed by organic extraction and alcohol precipitation of the RNA as decribed by Chomczynski (1993). 0.1 g of *T. tenax* cells were homogenised on ice in 1 ml TRIzol Reagent using a hand-held glass-teflon homogeniser and incubated for 5 min at RT. After the addition of 200 µl chloroform, the homogenate was mixed vigorously for 15 sec, incubated for 2–3 min at RT and centrifuged (12,000 x *g*, 15 min, 4°C). The upper aqueous phase (approximately 400 µl) containing the RNA was transferred to a fresh tube.
After this step the RNA was either precipitated by the addition of isopropanol or the sample was applied on a spin column and further treated using the RNeasy Mini Kit to obtain a higher purity and quantity of RNA, e.g. for cDNA synthesis (see 2.7.5).

Isopropanol precipitation
RNA was precipitated from the aqueous phase (400 µl) by mixing with 500 µl isopropanol and incubated for exactly 10 min at RT. After centrifugation (12,000 x *g*, 10 min, 4°C) the supernatant was discarded, the RNA was washed by adding 100 µl 70% ethanol, centrifuged (7,500 x *g*, 5 min, 4°C) and stored in 100% ethanol at -80°C.

RNeasy Mini Kit (Qiagen)
The aqueous phase (400 µl) was mixed with 700 µl RLT buffer containing 0.1 % β-Me and 2 ml ethanol was added. The sample was applied on the spin column and centrifuged (10,000 x *g*, 30 sec, RT). To eliminate contamination with genomic DNA, an on-column DNase treatment was performed to the manufacturer's instructions with slight modifications. The on-column DNase treatment was performed for 1 h at 28°C. The subsequent wash steps were carried out according to the manual instructions. The RNA was finally eluted from the column by applying 25 µl of RNase-free water, incubated for 1 min at RT and centrifuged (10,000 x *g*, 1 min, RT). This elution procedure was repeated and in a third step, 30 µl of eluate was applied on the column, incubated and centrifuged in order to improve RNA recovery.

The RNA samples were checked for residual DNA contamination by PCR amplification using 1 µl of the RNA preparation as template and primer set TtxC-2f and TtxC–2rev (T_m 60°C). When a PCR product (840 bp) was obtained, the respective RNA preparation was excluded from further microarray analyses and the preparation was repeated.

2.6.3 Quantitative and qualitative analysis of RNA

The concentration of extracted RNA was determined photometrically at λ = 260 nm. Absorption (A_{260}) = 1 corresponds to 40 µg RNA/ml (Sambrook *et al*., 1989).
The A_{260}/A_{280} ratio gives an estimate of RNA purity and sufficient pure RNA preparations showed a ratio greater than 1.8. Ratios less than 1.8 indicated contamination with protein or phenol.
The integrity of purified RNA was checked by denaturing formaldehyde agarose gel electrophoresis and ethidium bromide staining (see 2.6.4) as well as Northern blotting and methylene blue staining (see 2.6.5). The ribosomal RNA appeared as distinct bands (see 3.1.4).

2.6.4 Denaturing agarose gel electrophoresis of RNA

Electrophoretic separation of RNA was achieved under denaturing conditions in agarose-MOPS/formaldehyde gels (Staynov *et al*., 1972). For 1.2% (w/v) MOPS/formaldehyde gels, 1.2 g agarose was added to 73.8 ml DEPC treated water and 10 ml 10 x MOPS buffer (10 x: 200 mM morpholino propane sulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA, pH 7.0) and microwaved for 2 minutes. After cooling down to approximately 60°C, 16.2 ml of 37 % (v/v) formaldehyde was added and the agarose-formaldehyde gel was poured into the gel tray, and a comb (7-12 wells) placed into the tray slot. The solidified gel was placed in the electrophoresis chamber and completely covered with 1 x MOPS buffer.
The RNA samples and the RNA size marker (RNA Ladder, high Range) were mixed with 3 volumes of freshly prepared formaldehyde loading dye (250 µl deionised formamide, 83 µl 37% (v/v) formaldehyde, 50 µl 10 x MOPS buffer, 2.5 µl 2% (w/v) bromophenol blue and 14.5 µl DEPC treated water), incubated for 10 min at 65°C and finally chilled on ice.

Between 0.2-10 µg RNA and 50-100 ng RNA marker was loaded onto the gel. Electrophoresis was performed at 75 – 100 V for 1 – 3 hours depending on the gel size.
A rapid check for RNA integrity was performed by running an agarose gel (1.2%) containing EtBr (0.5 µg/ml; see 2.5.5).

2.6.5 Capillary transfer of RNA to a nylon membrane (Northern blot)

The electrophoretically separated RNA (see 2.6.4) was transferred from the agarose-formaldehyde gel to a positively charged membrane (Nytran) by capillary transfer. Therefore, the agarose gel was equilibrated in 20 x SSC buffer (3 M sodium chloride, 0.3 M sodium citrate, pH 7) for 2 x 15 min at RT. The membrane was wetted for 1 min with DEPC treated water and then soaked together with three Whatman filter papers (10 x 5 cm) in 20 x SSC buffer. The blot was assembled as shown in figure.2.2 and the transfer was performed overnight at 4°C.
The blot was quickly washed with DEPC-H_2O to remove salt from the membrane, then the RNA was UV-crosslinked (λ = 254 nm) for 3 min. Methylene staining was performed to visualise the immobilised RNA. The blot was swayed for at least 1 min in 50 ml staining solution containing 50 mg methylene blue, 6.6 ml 3 M sodium acetate, pH 5.2, 1 ml acetic acid (100%) and DEPC-H_2O. Destaining was achieved by washing the membrane 3–4 times with DEPC treated water.

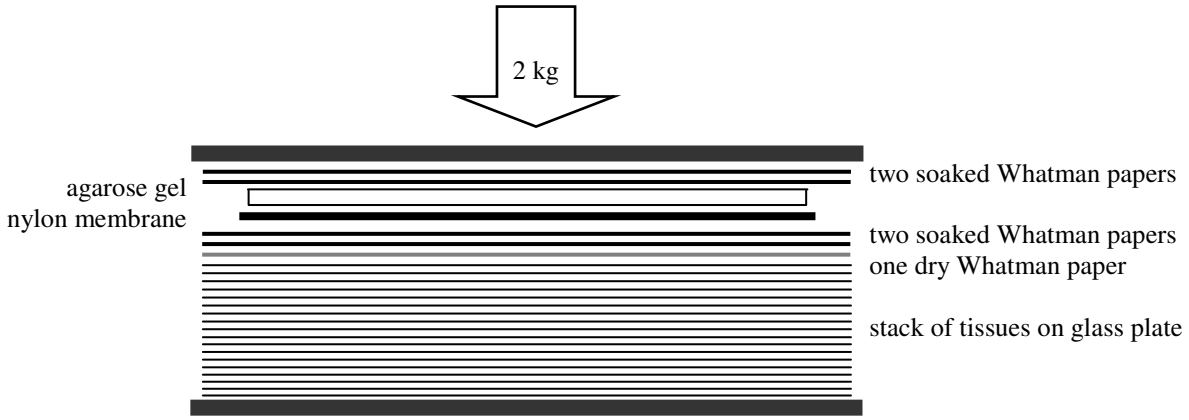


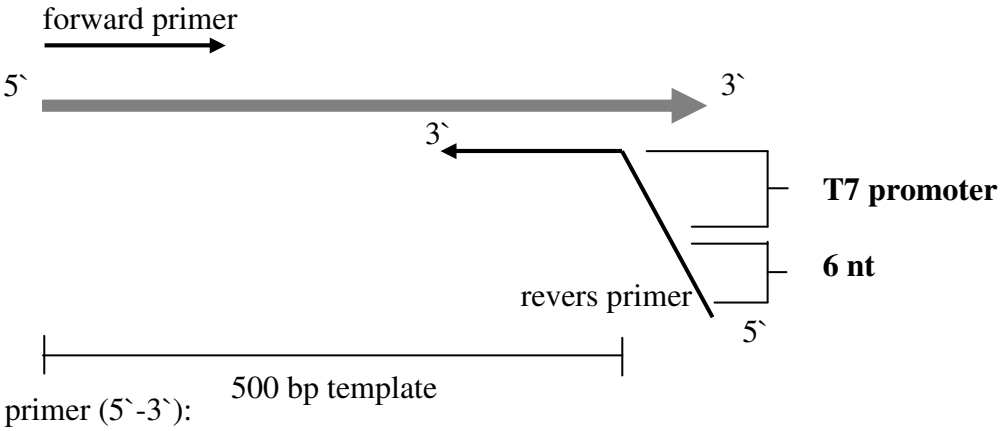
Fig. 2.2 **Blot assembly for transfer of RNA from an agarose gel to a positively-charged membrane (Northern blot).**

2.6.6 Hybridisation of immobilised total RNA with radioactively labelled specific RNA probes

Total RNA obtained from heterotrophically and autotrophically grown cultures (see 2.6.2) harvested in exponential growth phase was separated via denaturing formaldehyde agarose gel electrophoresis (see 2.6.4). After capillary transfer to a positively charged nylon membrane and UV-crosslinking (see 2.6.5), the RNA was hybridised with radiolabelled antisense RNA probes of eight selected ORFs (TTX_0910, *pps*; TTX_1105, *frdB*; TTX_0209, *oorA*; TTX_1513, *glt-A*2; TTX_1277, *pfp*; TTX_0864, *gpma*; TTX_1158, *gaa*) and of the ribosomal RNA, which was used as internal standard to assure equal amount of total RNA on the blot. Probe generation was carried out as follows (see 2.6.6.1).

2.6.6.1 Generation of specific, [α-^{32}P]-labelled antisense RNA probes by *in vitro* transcription

PCR products of the eight selected genes (about 500 bp) and the rRNA were amplified using *Taq* polymerase (100 µl reaction volume), purified after gel electrophoresis, quantified photometrically (see 2.5.4-2.5.7) and finally used as templates for probe generation and labelling via *in vitro* transcription. A sequence-specific forward and a modified reverse primer were used for the amplification. The reverse primer was constructed comprising the T7 binding site (5'-TAATACGACTCACTATAGGG-3') and additional six nucleotides (5'-GGGCCC-3') for T7 polymerase binding (see fig. 2.3 and tab. 2.5).



Reverse primer (5`-3`):
5`- GGGCCC**TAATACGACTCACTATAGGG** + specific sequence - 3`
6nt T7

Fig. 2.3 **Strategy of template generation for *in vitro* transcription.** PCR amplification was performed using sequence specific forward primer and a revers primer sequence (shown below and see tab. 2.5) containing T7 binding site (bold) and additional six nucleotides for polymerase binding (underlined).

Tab. 2.5 **Primer sets of template generation for *in vitro* transcription.**

Primer	sequence (5'-3')	T_m
*glt-A*2-T7-f	GGCGCGTTCGATGAAGATAA	55°C
*glt-A*2T7-fII	ATGAGCGAACAGACTGTATC	50°C
*glt-A*2T7-rev	GGGCCC**TAATACGACTCACTATAGGG**CGGTGTCCTACGCCCATCAG	57°C
frdB-T7-f	GTGAAACGATATAGAGACGGAGC	52°C
frdB-T7-rev	GGGCCC**TAATACGACTCACTATAGGG**TTAAAGCTCGGCGATGAGAT	52°C
gaa-T7-f	CTTGTATCTCAGTCGTTGGCGGT	62°C
gaa-T7-rev	GGGCCC**TAATACGACTCACTATAGGG**TCGCCGCCGCCCTCAGCCCGG	72°C
gpma-T7-f	GGGCTTGATGGCCAAAATAG	54°C
gpma-T7-rev	GGGCCC**TAATACGACTCACTATAGGG**AGCTGTACAGCTAGCTTTACAGCC	53°C
pfp-T7-f	GAGAGCAACGTGAAGGAGCTTGG	59°C
pfp-T7-rev	GGGCCC**TAATACGACTCACTATAGGG**CTCTCTATATACTCCGCCAGCTC	55°C
pps-T7-f	TATGGAGCAGGAGGGCCCAG	58°C
pps-T7-rev	GGGCCC**TAATACGACTCACTATAGGG**CGGGCTCGCCCAGATTCATA	59°C
Arch2-21-T7-f	TTCCGGTTGATCCYGCCGGA	55°C
Arch1510-1492-T7-rev	GGGCCC**TAATACGACTCACTATAGGG**GGTTACCTTGTTACGACTT	52°C

In vitro transcription

In vitro transcription was performed by using the T7 *in vitro* transcription kit (Fermentas Life Sciences) according the manufacturer's instructions. A standard reaction assay (20 µl) contained:

100-200 ng purified PCR product,
0.5 mM rATP, rGTP, rUTP,
50 µCi [α-^{32}P]-CTP (400 Ci/mmol), 6.25 µM final concentration
20 U T7 RNA polymerase,
4 µl Reaction buffer (5 x),
20 U RNase inhibitor

in DNase- and RNase-free H_2O.

The samples were incubated for 20 min at 37°C. The reaction was stopped by the addition of 2 µl 0.2 M EDTA, pH 8 and incubated for 10 min at 65°C. The *in vitro* transcribed antisense RNA was cleaned up using the RNeasy Mini kit and on-column DNase I digestion (Qiagen)

was performed according to the instructions of the supplier (also see 2.6.2). For the final elution step, 50 µl RNase-free H_2O was used, and a second elution step with 30 µl of the eluate was performed to higher the concentration of the sample.

Quantitation of probe labelling

Radioactivity was determined by quantifying 1 µl of the samples by liquid scintillation counting. Samples were combined with Rotiszint scintillation cocktail (10 ml) and counted using a Wallac Liquid Scintillation Counter (Model 1409). The probes were either directly applied for hybridisation (10^5 –10^6 cpm/ml hybridisation solution) or stored at -80°C.

2.6.6.2 Hybridisation of RNA with [α-^{32}P]-CTP labelled probes

Pre-hybridisation and hybridisation of the RNA blots were carried out in UltraHyb solution. The solution (15 ml/100 cm^2) was pre-heated and the blots were pre-hybridised for at least 1 h at 68-70°C in a hybridisation oven on a rocking platform (horizontal agitation). For the following hybridisation, the probes were denatured by incubation at 100°C for 10 min, quickly chilled in an ice/ethanol bath and applied to 15 ml of fresh, pre-heated UltraHyb solution. Hybridisation was carried out overnight.

Unbound probe was removed by stringency washes using varying buffers and temperatures. First washes were performed 2 x 5 min at RT using low-stringency buffer (2 x SSC, 0.1% SDS) to remove the hybridisation solution and not hybridised probe. The following high-stringency washes (0.5-0.1 x SSC, 0.1% SDS) were performed at 68°C-70°C for 2 x 15 min, to remove partially hybridised molecules.

2.6.6.3 Detection of RNA-RNA hybrids

The detection of the RNA-RNA hybrids was carried out by autoradiography. Therefore, the blots were wrapped in cellophane and exposed to a standard X-ray film (BioMax ML, 18 x 24 cm) to get a rough information of experimental success and guided to adequate exposure times for the following phosphorimaging autoradiography (sensitivity >24 x). Exposure was carried out at -80°C in a light-tight BioMax cassette equipped with a BioMax MS intensifying

screen. The films were manually processed in the darkroom using X-ray developer and fixer solutions (LX24 and AL4).

For phosphorimaging autoradiography, the exposure was carried out in BAS cassettes with an imaging plate (2025, 18 x 24 cm) at -80°C and empirically determined exposure times. Imaging and analyses was performed using a phosphorimager (Image Reader FLA 5000, V2.1). Data processing and quantitation was performed using AIDA software.

2.7 Design, fabrication and application of cDNA microarrays

2.7.1 Microarray probe generation using PCR

The gene sequences of 105 ORFs were obtained from the *T. tenax* genome (Siebers *et al*., 2004) and are listed in table 3.1 and 3.2. A total of 111 gene sequences of the 105 *T. tenax* ORFs were generated to be printed on the array. The oligonucleotide primer pairs (111; see appendix tab. A1) for the amplification of the microarray probes were designed using PrimeArray0.82 software (Raddatz *et al*., 2001; gained from Prof. C. Dehio, University of Basel) allowing for highly automated primer pair optimisation. The oligonucleotides were purchased from MWG Biotech. PCR amplifications were performed using HotStart *Taq* polymerase and Q solution (Qiagen) following the manufacturers' advice. The HotStart *Taq* polymerase was employed to improve amplification specificity. The enzyme was supplied in an inactive state, which prevents extension of non-specifically annealed primers and primer-dimers formed at low temperatures during PCR set-up. The enzyme required 15 min pre-heating at 95°C for activation.

The probes were generated in two rounds of hot-start PCR (500-1,000 bp; unless ORFs were < 500 bp; see tab. 3.1) in thin-wall PCR tubes. The initial round of PCR (25 µl reaction mixture) was performed using 50 ng genomic DNA as template, 1 µM of each primer, 1.25 U HotStart *Taq* polymerase, 1.5 mM MgCl, 200 µM dNTP mix, Q solution (1x) in accordant reaction buffer (1x), resulting in the "master set" of PCR products. 0.5-1µl of this master set served as template for the second round (100 µl reaction mixture) producing the "working set" of PCR products to achieve a sufficient dilution of genomic DNA in the samples. 2 µl of the PCR products of the master and the working set were checked by agarose gel electrophoresis (see 2.5.5 and figure 3.1). For those samples containing non-specific

amplified DNA, PCR conditions were optimised, e.g. by varying the annealing temperature, by adding DMSO, or gel-excised and purified (see 2.5.6), and specific DNA was re-amplified. The working set (the remaining 98 µl) was precipitated by the addition of 10 µl potassium acetate (pH 8.4) and 110 µl isopropanol overnight at -20°C. After centrifugation (4,000 x *g*, 3 h, 4°C) the supernatant was carefully discarded, 200 µl 70% ethanol was added and the samples were incubated for 1 h at -20°C. After centrifugation (4,000 x *g*, 1 h, 4°C) the supernatant was removed and the pellet dried under vacuum (speed vac).

The pelleted DNA was dissolved in 12 µl spotting solution 1 (150 mM sodium phosphate buffer, pH8.5, 0.01% SDS) overnight at 4°C. 2 µl were used for quantitation (A_{260}, see 2.5.4; appendix tab. A1). Finally, 10 µl of spotting solution 2 (150 mM sodium phosphate buffer, pH8.5, 0.01% SDS) containing 3 M betaine was added, resulting in a total volume of 20 µl probe [$\geq$ 100 ng/µl] in 0.15 M sodium phosphate buffer containing 0.01% SDS and 1.5 M betaine. The PCR products were stored at -20°C.

2.7.2 Printing of the microarrays

The dissolved PCR products (111, 20 µl; see 2.7.1) were pipetted onto microtitre plates. The spotting was performed with an Microgrid II robot (Biorobotics) using four pins (Microspot 2500 Pin for 96/384, BioRobotics) that had previously been checked for accuracy to prevent differences between the spots.

The PCR products (about 1.2 nl/spot) were printed six-fold and evenly distributed on poly-lysine-coated glass slides (Poly-Prep™; Sigma Diagnostics). On each slide, 28 fluorescence marker spots (Cy3 dye), for subsequent slide processing using GenePix software (see 2.7.7) and eight evenly distributed spots of PCR product of the *rpos* gene for internal standardisation, were additionally printed on the microarray. Furthermore, various spots of spotting buffer (about 500).

For long-term storage, the microtitre plates containing the remaining, non-spotted DNA were frozen at -20°C. The printed microarray slides were stored in a lightproof box at RT.

2.7.3 Post-processing of the slides

After spotting, the slides were left at RT overnight. Subsequently, the positively-charged surface of the slides were processed to deactivate remaining, unreacted amino residues of the poly-L-lysine to avoid unspecific cDNA binding during hybridisation (see 2.7.6).

Drying and UV-crosslink

The slides were dried (1 min, 80°C) array side up, on an inverted metal block from a standard benchtop heating unit and UV-crosslinked with a total energy of 60 mJ.

Blocking using succinic anhydride

Blocking of the slide surface was obtained by incubating the slides in 200 ml blocking solution, containing 50 mM succinic anhydride and 150 mM 1-methylimidazol in 1.2-dichlorethane, for 1 h at RT (Eisen and Brown, 1999). The succinic anhydride was removed by washing in 1.2-dichlorethane. The slides were transferred into boiling water for 2 min, then submerged in 96% ethanol. The slides were dried by centrifugation (400 x *g*, 5 min) at room temperature.

2.7.4 Preparation of the internal standard (*rpoS*) by *in vitro* transcription

The *rpoS* gene of *E. coli* coding for the stationary phase sigma factor was used as an internal standard. The *rpoS* gene was cloned by Dr. A. Zaigler, (formerly Goethe-University, Frankfurt) using the PCR-Script Amp cloning kit. The constructed vector (p*rpoS*) was transformed into *E. coli* K12 DH5α, plated onto ampicillin-containing agar plates and cultured for plasmid preparation (see 2.5.2). 5-10 µg of p*rpoS* was digested with 1 µl *Not*I (10 U/µl) at 37°C for 2 h. 1-2 µg of the purified *Not*I-linearised plasmid was used as template for the *in vitro* transcription assay using the T7 RNA polymerase kit (Fermentas), following the instructions of the manufacturer. A standard in vitro transcription assay (20 µl) for *rpoS*-RNA generation comprised:

1-2 µg	*Not*I-digested p*rpoS*,
0.5 mM	rGTP, rATP, rCTP, UTP,
4 µl	Reaction buffer (5x),
0.7 µl	RNase inhibitor [25 U/µl],

1 µl	T7-RNA polymerase [20 U/µl],

in DNase- and RNase-free H_2O. The reaction mixture was incubated for 2 h at 37°C. 2 µl RNase-free DNase I was added and incubation was continued for 15 min at 37°C to digest the DNA template. The reaction was stopped by the addition of 2 µl 0.2 M EDTA and the generated RNA was precipitated by adding 2.5 µl LiCl (4 M) and 75 µl 100% (v/v) chilled ethanol and incubation for 2 h at -20°C. After centrifugation (12,000 x *g*, 15 min, 4°C), the pellet was washed with 50 µl chilled 70% (v/v) ethanol and centrifuged again (12,000 x *g*, 15 min, 4°C). The supernatant was discarded, the open tube was placed in a benchtop heating unit for 5 min at 37°C to remove traces of ethanol and finally the RNA was dissolved in 50 µl DEPC-H_2O. The integrity of the *in vitro* generated RNA (*rpoS*) was controlled by formaldehyde agarose gel electrophoresis (see 2.6.6) and Northern blotting (see 2.7.7). The concentration was photometrically determined at $\lambda = 260$ nm.

RNA preparations from autotrophically and heterotrophically grown cells were made up as described previously (see 2.6.2) and identical amounts (40 ng) of *in vitro* transcribed *rpoS* were added to each RNA preparation sample immediately after cell lysis. The RNA was stored at -80°C.

2.7.5 Target generation: Labelling and cDNA synthesis of total RNA from *T. tenax*

Fluorescently labelled cDNA was prepared by reversed transcription via reverse transcriptase (M-MLV RT (RNase H Minus)) from 15 µg of total *T. tenax*-RNA and 40 ng *rpoS*. Total RNA obtained from autotrophically grown cells was labelled using Cy3-dUTP (green) and heterotrophic RNA was labelled with Cy5-dUTP (red) fluorescence dye. The standard cDNA synthesis assays (40 µl) comprised:

15 µg	RNA,
0.6 µl	Hexamer mixture [1 mg/ml],
0.5 mM	dATP, dCTP, dGTP,
0.2 mM	dTTP,
0.0625 mM	Cy3-dUTP or Cy5-dUTP, respectively,
8 µl	Reaction buffer (5 x)

in RNase- and DNase-free H2O. After incubation at 65°C for 5 min, 2 µl of the reverse transcriptase (RT) [200 U/µl] was added and RT reaction was performed at 42°C for 2 h and finally stopped with of 5 µl 0.5 M EDTA. By the addition of 10 µl 1 M NaOH, followed by

further incubation at 65°C for 1h, the RNA template was degraded. To neutralise the reaction mixture 25 µl Tris/HCl (1M, pH 7.5) was added and the differentially labelled cDNA (derived from auto- and heterotrophically grown *T. tenax* cells) were pooled and DNase-free H_2O was added to a volume of 500 µl. The sample was purified and concentrated to 50 µl by using Microcon YM-30 centrifugal filter units (Millipore). Again, DNase-free H_2O (450 µl) was added and the samples were concentrated to a volume of 17 µl using Microcon YM-30. Finally 1µl [4 mg/ml] yeast-tRNA was mixed into the sample to competitively block the poly-lysine surface and therefore reduce the background.
The samples containing the differentially labelled cDNA of auto- and heterotrophically grown cells, was either directly used for hybridisation or stored at -20°C.

2.7.6 Hybridisation of the labelled cDNA to the microarray

Prior to hybridisation of the labelled cDNA to the probe DNA on the microarrays, the slides were pre-hybridised in pre-warmed 3.5 x SSC containing 0.1% SDS and 10 µg/ml BSA at 50°C for 25 min, washed for 30 sec in aqua bidest. and 30 sec in isopropanol, and finally dried by centrifugation (500 rpm, 5 min, RT).
For hybridisation, the cDNA sample was mixed with 3.75 µl 20x SSC and 1.25 µl Denhardt's solution (50 x: 1% Ficoll 400 (w/v), polyvinylpyrrolidone (w/v) and bovine serum albumin (w/v)). The hybridisation mixture (23 µl) was heated at 98°C for 2 min to denature the cDNA and 2µl 1% SDS was added. The hybridisation solution was pipetted on the microarray slide and a coverslip (Hybri-Slip, 22 x 22 mm) was placed on top. The slides were transferred in a Corning CMT hybridisation chamber and hybridisation was performed at 60°C over night.
Afterwards, the slides were washed twice in the dark in 0.5 x SSC containing 0.01 % SDS and in 0.06 x SSC for 5 min each. The slides were dried by centrifugation (400 x *g*, 5 min, RT).

2.7.7 Scanning of the microarrays, data processing and analysis

Scanning of the microarrays and measuring the excitation of Cy3 and Cy5 at 532 and 635 nm, respectively, was performed using a GenePix 4000a scanner (Axon Instruments).
Image processing and spot analysis was performed using GenePixPro 3.0 software (Axon Instruments). Initially, an automatic spot finding and quantification software tool was used.

Subsequently, each spot was edited by hand to determine their intensity and homogeneity. In some cases the spot diameter of the automatic spot finding tool was corrected. Low-quality spots (<1,000 intensity units, inhomogeneous shape) were excluded from further analysis. Low-intensity signals show a high standard deviation because of background fluorescence. Generally, spots were excluded in case of local high background signal.

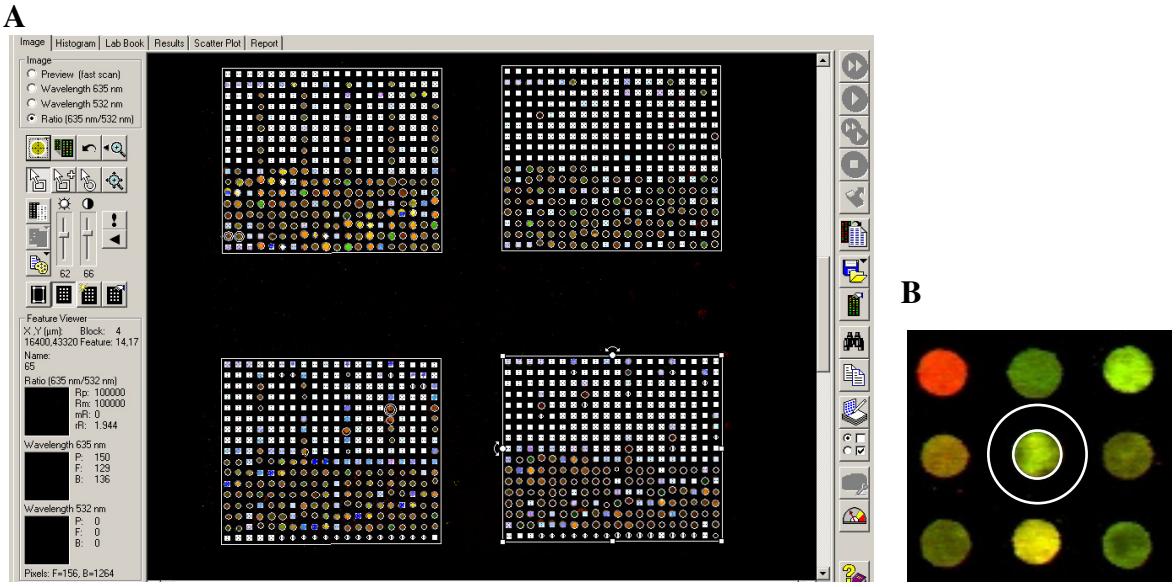


Fig. 2.4 **Image processing and spot analysis using GenePixPro3.0 software.** After initial automatic spot finding (**A**), the quantification tool of the software was used to analyse local neighbourhood for background correction (**B**). Subsequently, each spot was edited by hand concerning intensity and homogeneity.

The obtained signal intensities were normalised by using the method of internal standardisation, as reported previously by Zaigler and coworkers (Zaigler *et al*., 2003). The *rpoS* gene of *E. coli* served as the internal standard. The average Cy3 and Cy5 signal intensities of the eight *rpoS* spots (see 2.7.2) were determined and used for normalisation. This normalisation method compensates methodical differences, e.g. due to differential dye incorporation.

A control experiment was performed to test the reliability of the obtained data and the reproducibility of the procedure. For this purpose, cDNA derived from two independent autotrophically grown cultures was hybridised and analysed (see 3.1.4).

The red/green (Cy5/Cy3) ratios of the corrected median pixel intensities are presented as log2 values and standard deviation (SD) is given. Statistical significance for the observed ratios was obtained by *t*-test analysis (significance level $P < 0.05$) using GeneSpring software and Microsoft Excel (paired *t*-test). Genes with a significance level of $P < 0.05$ were accounted as

statistically significant and those genes that showed expression changes about twofold (and with a significance level of $P < 0.05$) were accounted as differentially expressed genes.

2.8 Biochemical methods

2.8.1 Heterologous expression of the *T. tenax* TPSP, GT, Msc, LrP1 and HP5 in *E. coli*

For the heterologous expression of the *T. tenax* TPSP (trehalose-6-phosphate synthase/ phosphatase), GT (glycosyl transferase), Msc (mechanosensitive channel), LrP1 (leucine-responsive regulatory protein 1) and HP5 (hypothetical protein 5) the encoding *tpsp* (TTX_1304), *gt* (TTX_1305), *msc* (TTX_1304a), *lrp1* (TTX_1154) and the *hp*5 (TTX_1155) genes were cloned using the T7 polymerase pET vector system via restriction sites introduced by PCR mutagenesis (for respective primer sets see tab. 2.2; for nucleotide and amino acid sequence see appendix (Fig. A1 and A2). PCR mutagenesis was performed using *Pfu* polymerase, genomic DNA of or a genomic clone (H88; A. Brenner, 2001) of *T. tenax* served as template. The sequences of the cloned genes were verified by dideoxy sequencing (see 2.5.10).

Heterologous expression of the recombinant enzymes was performed in *E. coli* BL21(DE3), BL21(DE3) CodonPlus (pRIL), Rosetta(DE3) and BL21(DE3)pLysS as discussed previously (see 2.3). These *E. coli* strains contain a λ prophage (DE3), carrying the T7 RNA polymerase gene and the T7*lac* promoter. In the transformed pET vector constructs the cloned genes are under control of the T7*lac* promoter and expression is repressed, until IPTG induction of the T7 RNA polymerase from the lac promoter.

The pLysS plasmid codes for T7 lysozyme, a natural inhibitor of the T7 polymerase, which is constitutively expressed. Therefore, the *E. coli* strain BL21(DE3)pLysS is used to minimise low-level expression of potentially toxic gene products before IPTG induction. The strain was used for the heterologous expression of the MscTTX in *E. coli*.

The putative MscTTX was also cloned into the vector pTrcH6 (kindly provided by Prof. Dr. Ian Booth, University of Aberdeen (Scotland, UK).

2.8.2 Expression of the *T. tenax* MsC in *Sulfolobus solfataricus*

For the heterologous expression of the *T. tenax* MscTTX in *S. solfataricus,* the *msc* gene was cloned via restriction sites introduced by PCR mutagenesis (see tab. 2.2) into the vector pMZ1, kindly provided by Dr. S.V. Albers, University of Groningen, containing an arabinose-inducible promoter of the arabinose-binding protein AraS and a streptavidine-histidine-tandemtag (Albers *et al*., 2006). PCR mutagenesis was performed using *Pfu* polymerase and genomic DNA of *T. tenax* served as template for the PCR reaction. The sequence of the cloned *msc* gene was verified by dideoxy sequencing.
The following steps were performed by Dr. S.V. Albers, University of Groningen (NL): The preconstruct pMZ1-*msc* was *Bln*I/*ApaI* restricted and cloned in frame with the tandem (6x histidine-streptavidine)-tag into to the *Bln*I/*ApaI* restricted shuttle vector pMJ03 (Jonuscheit *et al.*, 2003, modified; Albers *et al.*, 2006) resulting the expression plasmid pSVA80 and electroporated *S. solfataricus* PH1-16 (Δ*pyrEF*) cells were transformed with the plasmid (Jonuscheit *et al.*, 2003; Schleper *et al.*, 1992). The cells were grown in 5 l selective medium (lacking uracil; see 2.3). Induction was performed by the addition of 0.4% arabinose (at OD_{600} 0.3), expression was carried out for 12 h and the cells were harvested by centrifugation.

2.8.3 Preparation, enrichment and purification of the recombinant enzymes

2.8.3.1 Enrichment of the recombinant TPSP

Recombinant *E. coli* Rosetta(DE3) pET24a-*tpsp* cells were resuspended in chilled lysis buffer (100mM Tris/HCl, pH 7 (70°C), 7.5 mM DTT; 3 ml/g cells) and passed three times through a French pressure cell at 20,000 psi. Cell debris and unbroken cells were removed by ultracentrifugation (60,000 x *g*, 45 min, 4°C). The cell pellet was diluted in lysis buffer (10 ml/g) and kept for subsequent SDS PAGE analysis at 4°C or at -20°C in Laemmli buffer. For enrichment, the supernatant was diluted (1:1) with lysis buffer and subjected to heat precipitation at different temperatures (60-90°C) for 20 min to remove unwanted *E. coli* proteins. After heat precipitation, the samples were centrifuged (50,000 x *g*, 30 min, 4°C) and dialysed overnight at 4°C against 100mM Tris/HCl, pH 7 (70°C), 7.5 mM DTT containing 5 mM $MgCl_2$. The enriched protein was directly used for the enzymatic assays (see 2.8.4).

Phosphorylation of the TPSP was checked by incubating 10 µl of heat precipitated fraction at 70°C (HP70) in an assay containing 100 mM Tris/HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 10 µl and 2 U alkaline phosphatase (CIAP; Ahmad and Huang, 1980, modified). The samples were incubated for 20 min at 37°C and subsequently analysed by SDS-PAGE.

2.8.3.2 *In vitro* reconstitution of the GT from inclusion bodies

Recombinant *E. coli* BL21(DE3) pET24a-*gt* and Rosetta(DE3) pET24a-*gt* cells were resuspended in 100mM Tris/HCl, pH 7 (70°C), 7.5 mM DTT or 100 mM HEPES/KOH, pH 7.5 (RT), 100 mM NaCl and 50 mM DTT lysis buffer (3 ml/g cells). The preparation of crude extract and heat precipitation was carried out as described above (see 2.8.3.1).
Due to a very low amount of recombinant protein in the soluble fraction, the GT was purified from inclusion bodies, which are insoluble, inactive aggregates that are often formed during high-level production of recombinant proteins in *E. coli*. Inclusion bodies contain the respective protein in a highly enriched form and can be isolated by *in vitro* refolding techniques. Hereunto, 5 g recombinant cells were resuspended in 20 ml 100 mM HEPES/KOH, pH 7.5 (RT), 100 mM NaCl and 50 mM DTT lysis buffer (buffer A), passed three times through a French pressure cell at 20,000 psi. Due to the high density of the inclusion bodies, they can be pelleted by centrifugation (4,500 x *g*, 10 min, 4°C) and thus, isolated from the lysate. The supernatant was discarded and the pellet resuspended in 20 ml buffer A. After the addition of 0.5 % (v/v) Triton X-100, the sample was vortexed, 2 mg DNase I, 2 mg RNase and 4 mg lysozym were added and the sample was incubated for 1 h at 37°C. Subsequently, the sample was centrifuged (4,500 x *g*, 10 min, 4°C), the supernatant removed and the pellet resuspended in 20 ml buffer A containing 5 mM sodium deoxycholate, followed by intense vortex and centrifugation (4,500 x *g*, 10 min, 4°C). 10 ml of washed inclusion bodies thus obtained were dissolved in 90 ml buffer A containing 2 M guanidinium hydrochloride (GHCl) and stirred for 24 h at RT for denaturation (Umetsu *et al.*, 2003, modified).
The released protein was then refolded by removal of the denaturing agent by dialysis against buffer A for 12 h at RT (2 x). After centrifugation (12,000 x *g*, 15 min, 4°C) the samples were concentrated via Amicon spin column (< 30,000 kDa).
The reconstituted, recombinant GT was either directly used for the enzymatic assays (see 2.8.4), stored at 4°C or mixed with glycerol (final conc. 10% (v/v)) and frozen at -80°C for

long-term storage.

2.8.3.3 Isolation of the recombinant MsC from *S. solfataricus*

After expression and harvesting the cells, the membranes were solubilised and MscTTX containing the strep-his-tag was purified via His-tag specific affinity chromatography using His-Select column. After SDS-PAGE the protein was blotted and staining with Strep-Tactin (streptavidine analogue; see 3.3.6.2 and fig.3.28). This work was performed by Dr. S.V. Albers, University of Groningen (NL).

2.8.3.4 Enrichment of the recombinant Lrp1 and HP5 for EMSAs

Recombinant *E. coli* Rosetta(DE3) pET15b-*hp*5 and Rosetta(DE3) pET15b-*lrp1* cells were resuspended in TEK buffer (10 mM Tris/HCl, pH 8 (RT), 10 mM potassium chloride, 1 mM EDTA, 10 mM β-Me, 1 mM PMSF; 2 ml/g cells). The preparation of crude extract and heat precipitation (80°C for 30 min) was carried out as described in section 2.8.3.1.
The protein was dialysed against TEK buffer overnight at 4°C. The samples were either directly used for the EMSA experiments, stored at 4°C or mixed with glycerol (final conc. 25 % (v/v)) and frozen at -80°C for long-term storage.
Phosphorylation of Lrp1 was checked by incubation 10 µl of heat precipitated fraction at 70°C (HP70) in an assay containing 100 mM Tris/HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 10 µl and 1 U alkaline phosphatase (CIAP; Ahmad and Huang, 1980, modified). The samples were incubated for 20 min at 37°C and subsequently analysed by SDS-PAGE.

2.8.3.5 Purification of His-tagged recombinant enzymes

The *T. tenax tpsp*, *gt*, *msc* and *lrp1* genes were additionally cloned in frame with an N- or C-terminal His-tag (6x histidine; see tab. 2.2), respectively, in order to purify and enrich recombinant proteins after heat precipitation from the soluble fraction.
His-tag specific affinity chromatography was performed using Ni-NTA agarose column. Prior to application of the protein (1-2 ml of the HP fractions, see 2.8.3.1), column was equilibrated

with the respective lysis buffer of the protein sample (20 ml buffer A: 100mM Tris/HCl, pH 7 (70°C), 7.5 mM DTT 100 mM HEPES/KOH, pH 7 (70°C), 7.5 mM DTT or 550 mM sodium phosphate buffer, pH 7.5 (RT) containing 300 mM sodium chloride). After washing (10 ml buffer A) elution was performed with 7-10 ml buffer B: BufferA containing 100-250 mM imidazole. Single fractions of 1 ml were collected of each step and subsequently analysed using SDS-PAGE.

2.8.4 Determination of the enzyme activities of the recombinant TPSP

The N-terminal TPS domain of the trehalose-6-phosphate synthase/phosphatase (TPSP) catalyses the formation of trehalose 6-phosphate (Tre6P) from UDP-glucose (UDPG) and glucose 6-phosphate (G6P). The C-terminal TPP domain catalyses the dephosphorylation Tre6P by forming trehalose (Tre):

TPS (EC 2.4.1.15): $UDPG + G6P \longrightarrow Tre6P + UDP$

TPP (EC 3.1.3.12): $Tre6P + H_2O \longrightarrow Tre + P_i$

Discontinous activity assay

The activity of the *T. tenax* TPSP was determined by using a discontinuous assay, which was carried out at 70°C in 100 mM Tris/HCl, pH 7 at 70°C in the presence of 4 mM UDP- or ADP-glucose, 8 mM glucose 6-phosphate, 4 mM $MgCl_2$ and 50 µg protein in a final volume of 125 µl. The assay was performed in presence (50 µg) and absence of the recombinant, putative GT. The activity of the TPP domain of the TPSP, as well as of the single TPP was measured at 50 and 70°C in 100 mM Tris/HCl, pH 7 at 50 or 70°C, respectively (Matula *et al*., 1971, modified). The assay contained 2 mM trehalose 6-phosphate, 4 mM $MgCl_2$ and 50 µg protein in a final volume of 125 µl.

The discontinuous TPS-assay (Hottiger *et al*., 1987; Page-Sharp *et al*., 1999, modified) was performed at 70°C in 100 mM Tris/HCl, pH 7 at 70°C in presence of 8 mM glucose 6-phosphate, 4 mM UDP- or ADP-glucose, 10 mM $MgCl_2$ and 60 µg protein in a final volume of 125 µl for 30 and 60 min.

The reactions were started by the addition of the proteins. After accordant incubation time at the respective temperatures, the reactions were stopped and protein was precipitated by the addition of acetone (1:1) and subsequent incubation at –20°C for 20 min.

Negative controls without protein, only one substrate, and cell-free extract of expression host with empty vector were performed.

Detection

The activities of the recombinant TPSP, TPP and TPS as well as the recombinant GT were qualitatively analysed by detection of the intermediates and products using thin layer chromatography (TLC; see 2.8.6).

2.8.5 Measurements in crude extracts of *T. tenax*

The activities of the TPSP and the GT were determined in crude extracts (CE) of *T. tenax* grown autotrophically and heterotrophically on glucose. Therefore, 1 g of the respective *T. tenax* cells was resuspended in 3 ml 100 mM HEPES/KOH, pH 7 (86°C) containing 10 mM β-Me and 5 mM $MgCl_2$, passed three times through a French pressure cell at 20,000 psi, then cell debris was removed by ultracentrifugation (60,000 x *g*, 45 min, 4°C). Half of the crude extracts were dialysed over night at 4°C against 100mM HEPES/KOH, pH 7 (70°C) containing 10 mM β-Me and 5 mM $MgCl_2$.

The heterotrophic and autotrophic discontinuous TPSP + GT assays contained 4 mM UDP-glucose, 8 mM glucose 6-phosphate, 4 mM $MgCl_2$ and 50 µg total protein in 100 mM Tris/HCl, pH 7 (86°C) containing 10 mM DTT in a final volume of 100 µl. The discontinuous TPP assays contained 2 mM Trehalose 6-phosphate, 4 mM $MgCl_2$ and 50 µg total protein in 100 mM Tris/HCl, pH 7 (86°C) containing 10 mM DTT (final volume 100 µl).

The reactions were carried out at 86°C for 60 min using dialysed and non-dialysed crude extracts of heterotrophic and autotrophic cells. Negative controls without substrate and crude extract were also performed. The substrates and intermediates were qualitatively analysed by thin layer chromatography (see 2.8.4).

2.8.6 Thin layer chromatography (TLC)

The chromatographic TLC technique is useful for separating small compounds. TLC was used for the qualitative detection of the components in the enzyme reaction assays to monitor trehalose formation by the recombinant *T. tenax* enzymes.

The adsorbent material of the stationary phase consisted of silica immobilised on glass plates (silica gel G60 plates). The solutions to be separated were dissolved in an appropriated solvent (100 ml) containing 50 ml butan-1-ol, 30 ml ethanol and 20 ml H_2O.
After incubation of the enzyme assays, the containing protein was precipitated by the addition of acetone (1:1) and incubation at -20°C for at least 30 min. After centrifugation (12,000 x *g*, 20 min, 4°C) the supernatant was transferred in a fresh tube and the samples were concentrated using speed vac centrifugation. 10-20 µl of the samples were spotted near the bottom of a silica plate and additionally 10 µl of standard solutions (10 mM glucose, glucose 6-phosphate, UDP/ADP-glucose, trehalose and trehalose 6-phosphate) were spotted. The plates were placed in a developing chamber comprising the solvent, which slowly rose up the plate by capillary action (over night, at RT) and, thereby, separated the components of the enzyme assays.
For signal detection the plates were dried, sprayed with 20% H_2SO_4 and subsequently incubated at 100°C for 15 min.

2.8.7 Analytical protein methods

2.8.7.1 Protein quantitation

The determination of protein concentration was carried out using Bio-Rad Protein Assay based on the Bradford protein quantitation method (Bradford, 1976; modified) and following the instructions of the supplier. Bovine serum albumin (BSA; 2-10 µg/ml) served as standard.

2.8.7.2 SDS Polyacrylamide gel electrophoresis (PAGE)

For protein analysis and separation, denaturing sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE) according to Laemmli (Laemmli, 1970) was used. Here, polyacrylamid (PAA) gels (8.6 cm x 7.7 cm, 1 mm thick) composed of a stacking (on top), and a separating gel were used as support matrix in electrophoresis. The acrylamide concentration of the stacking gel was 4.0% (v/v) (125 mM Tris/HCl, pH 6.8, 0.1% (v/v) SDS, 0.03% (v/v) APS, 0.005% (v/v) TEMED) and the concentration of the separation gel (375 mM Tris/HCl, pH 8.8, 0.1% (v/v) SDS, 0.03% (v/v) APS, 0.005% (v/v)TEMED) varied

between 7.5 and 15 % (v/v) polyacrylamide due to the molecular weight range of the protein subunits as well as the desired separation of the proteins.
Separating gel ingredients were mixed and poured in the gel casting chamber. The gel was covered with butan-1-ol and allowed to polymerise for 20 min. The butan-1-ol was then removed, the gel surface was washed with aqua bidest., dried with Whatman paper and the stacking gel solution was poured. A 12-sample well comb was placed into the gel, then removed after approximately 20 min. The gels were either directly used for electrophoresis or stored at 4°C.
Prior to electrophoresis, the protein samples were mixed with 2 x or 5 x loading buffer (final concentration: 62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (v/v) SDS, 5% (v/v) β-mercaptoethanol, 0.005% (w/v) bromophenol blue) and heated for 2–3 minutes at 94°C.
Gel runs were performed in a Minigel-Twin-Chamber (Biometra) containing electrophoresis buffer (25 mM Tris-HCl, 190 mM glycine and 0.1% (v/v) SDS) at 120 V.
Detection of the proteins was performed by gel staining (40% (v/v) methanol, 10% (v/v) acetic acid and 0.25% (w/v) Coomassie Brilliant Blue R-250) for 30 min at 50°C and destaining (5% (v/v) methanol and 7.5% (v/v) acetic acid) (Weber and Osborn, 1969). The gels were analysed using the ChemiDoc System in combination with Quantity One Software Package (BioRad).

2.8.7.3 Molecular mass determination under denaturing conditions

The approximate molecular mass of protein subunits was determined by SDS-PAGE using different standards:

Dalton Mark VII-L Standard Mixture (SDS-7) comprises a molecular mass range of 14.2–66 kDa (α-lactalbumin, bovine milk (14.2 kDa), trypsin inhibitor, soybean (20.1 kDa), trypsinogen, bovine pancreas (24 kDa), carbonic anhydrase, bovine (29 kDa), glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (36 kDa), egg albumin (45 kDa) and bovine albumin (66 kDa).

The standard SDS-6H ranges from 14.2 – 205 kDa (myosin, rabbit muscle (205 kDa), β-galactosidase, *E. coli* (116 kDa), phosphorylase B, rabbit muscle (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa) and bovine carbonic anhydrase (29 kDa)).

To monitor protein separation during SDS-PAGE and to monitor and verify protein transfer efficiency on membranes (Western blot, see 2.8.9) a prestained protein molecular weight marker with a range of 20 – 116 kDa was used (β-galactosidase, *E. coli* (116 kDa), albumin, bovine plasma (86 kDa), ovalbumin, chicken egg white (47 kDa), carbonic anhydrase, bovine erythrocytes (34 kDa), β-lactoglobulin, bovine milk (26 kDa), lysozyme and chicken egg white (20 kDa)). All six contained proteins are covalently coupled to a blue chromophore.

2.8.7.4 Electrotransfer of separated protein species to a membrane (Western blot)

For N-terminal sequencing, electrophoretic separated protein was transferred from the PAA gel to a hydrophobic membrane (PVDF ProBlott) by semi-dry electroblotting. The transfer was carried out using a semi-dry carboglass transfer apparatus.
Electrophoresis of the recombinant *T. tenax* TPSP (HP90°C fraction, see 2.8.3.1) was performed in a large 7.5% (v/v) PAA gel and 10 µl of prestained molecular weight standard (see 2.8.7) were used. After electrophoresis run, the stacking gel was removed from the separation gel and the latter was equilibrated in blotting buffer (10 mM CAPS, pH 11, 10% (v/v) methanol) for 5 min. The membrane was briefly moistened with 100% (v/v) methanol and afterwards equilibrated in blotting buffer. Blot assembly was performed as previously described (Jungblut *et al.*, 1990). Therefore, the membrane was placed on top of two wet Whatman paper laying on the anode, followed by the gel and finally two additional wet Whatman paper. The transfer was carried out with 1 mA/cm^2 for 3 h at RT.
After electroblotting the membrane was washed with H_2O and stained for 1 min in staining solution (40% (v/v) methanol, 1% (v/v) acetate, 0.1% (w/v) Coomassie Blue R-250). After destaining with 50% (v/v) methanol, the blot was washed and dried.

2.8.7.5 Determination of the N-terminal amino acid sequence

The determination of the N-terminal amino acid sequence of electrophoretically separated proteins, which were transferred to a hydrophobic membrane (Western blot) was carried out by automated Edman-degradation in a gas phase-sequencer (Proteinsequencer 473A, Applied Biosystems). The N-terminal sequencing was performed by Dr. R. Schmid of the Institute for Microbiology, University of Osnabrück, Germany.

3 RESULTS

3.1 Transcriptional profiling of the CCM genes using cDNA microarrays

The microarray was constructed harbouring 111 gene sequences of 105 ORFs mainly involved in the CCM of *T. tenax* (see 3.1.1). Sequence information of the CCM ORFs was derived from the *T. tenax* genome (Siebers *et al.*, 2004).
Seven hybridisation experiments with cDNA derived from 14 independent cultures of *T. tenax*, seven grown autotrophically on CO_2 as sole carbon source and seven grown heterotrophically on glucose (see 2.3 and 3.1.3) were performed, in order to analyse the response to the different carbon sources.
A control experiment with RNA derived from two independent autotrophically grown cultures was carried out in order to check for reproducibility of the microarray experiments and to underline reliability of the obtained data (see 3.1.4.1).

3.1.1 Microarray fabrication

For the fabrication of the CCM microarray the 111 probes of the 105 ORFs were generated via PCR amplification with a size range of 0.5 –1.0 kb and arrayed onto the polylysine glass slides. For the ORFs TTX_1762, TTX_1455, TTX_1316, TTX_1396 and TTX_1768 probes from the N- and the C-terminal part were generated, due to initial genome contig information.
The oligonucleotide primers were designed using PrimeArray0.82 software allowing for highly automated primer pair optimisation. Table A1 (see appendix) comprises all primer sets, their 5'-3' sequences and the respective probe sizes of the 111 sequences.

3.1.1.1 Probe generation

Master set of PCR products
To ensure a high PCR specificity of the probes, HotStart *Taq* polymerase was employed for PCR amplification. An initial incubation step at 95°C for 15 min was performed for activation of the polymerase and the PCR reaction, respectively (see 2.7.1). The size of the PCR products was checked by agarose gelelectrophoresis applying 4 µl of the Master set on an agarose gel (see fig. 3.1). For those candidates, which show no or unspecific amplification,

optimisation of the PCR conditions by altering the annealing temperature was conducted.

The amplification of the probes 10, 15, 35 and 72 revealed some problems. In case of probe 10, lowering annealing temperature yielded a low amount of PCR product that was purified and reamplified. Also sufficient amount of the probes 15 and 35 was achieved for reamplification by altering PCR conditions. For probe 72 no PCR product was observed and therefore, a new revers oligonucleotide primer was designed.

Finally a complete master set of PCR products of all 111 sequences (see fig. 3.1) of a sufficient concentration was obtained, that was used for working set generation.

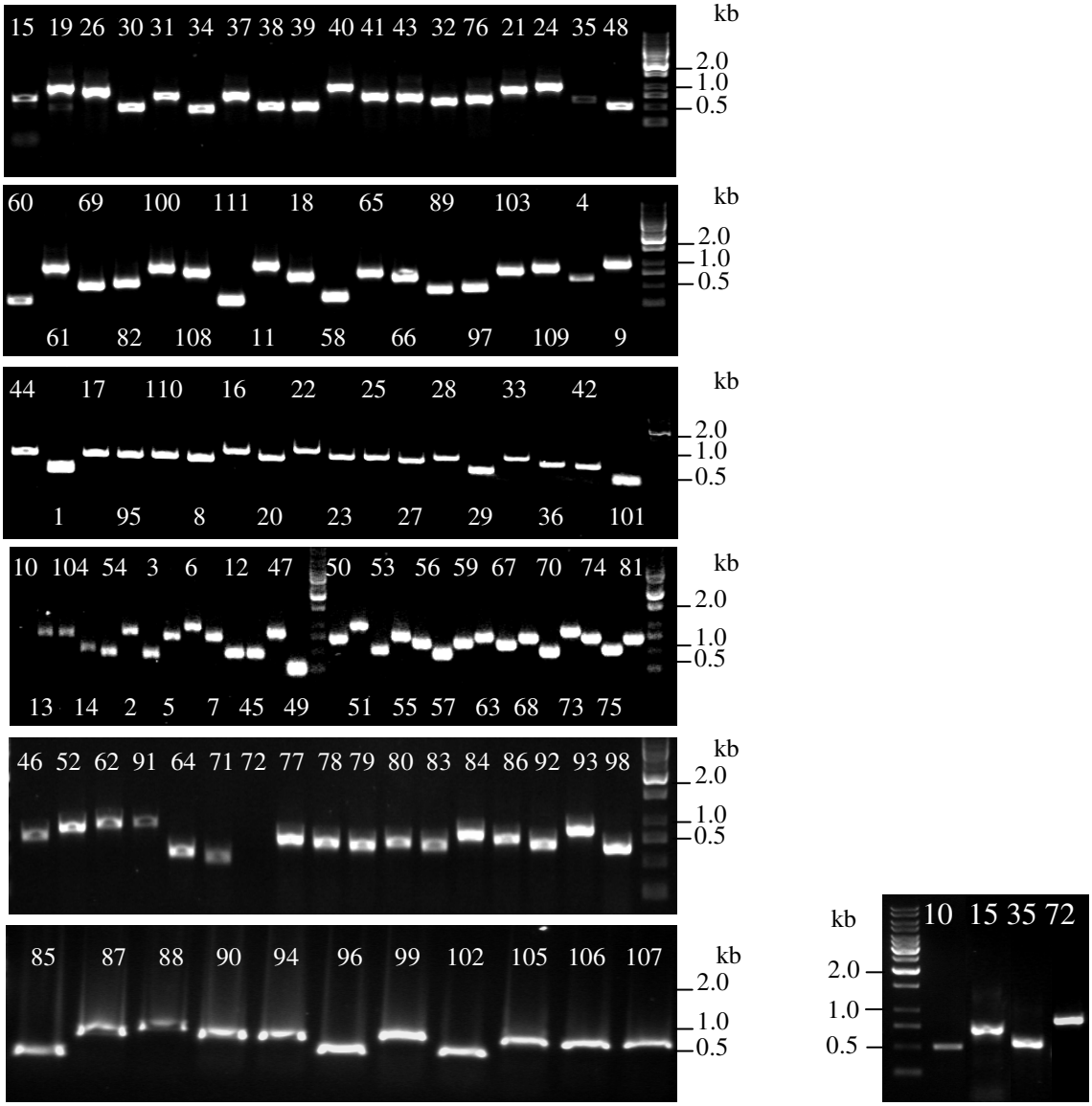


Fig. 3.1 **Agarose gel electrophoresis of the PCR products (probes).** Complete master set of 111 probes. Given numbers correspond to the numbering in table 3.1 (first column).

Working set of PCR products

The generation of the working set was performed by using 0.5 – 1 µl of the master set as template for PCR reaction. The reactions were carried out at the determined and optimised PCR conditions of master set production. All PCR products of the working set were also checked by agarose gel electrophoresis by applying 3 µl of the sample on an agarose gel.
Precipitation of the PCR products was carried out as described previously (see 2.7.1). The DNA was dissolved in spotting solution containing 1.5 M betaine and quantified spectrophotometrically at 260 nm. Sufficient concentration ($\geq$ 100 ng / µl) of each probe was obtained (see appendix tab. A1).

3.1.1.2 Printing and quality of the *T. tenax* microarrays

The microarrays (80 slides) were printed with a MicroGrid II spotter, performing one run over night. A total number of 1,280 spots per slide were arrayed. Each of the 111 sequences were printed sixfold and evenly distributed onto one slide, additional eight spots of *rpoS* probe, position marker (Cy3 dye) and negative controls, predominantly spotting buffer were arrayed (see fig. 3.4).
A total of 65 of the printed arrays proofed to be of good quality and these arrays were used for the microarray experiments. Some of the arrays (15) were excluded form further analyses due to irregular printing (spot merging, missing spots). The spotting solution left visible dots on the slides during arraying process, due to the salt contained in the spotting solution and thus, it was possible to control accurate printing of the pins. Prior to post-processing of the slides (see 2.7.3), the area of the spotted DNA was marked, because the dots vanish during processing. Subsequently, a random selected slide was checked by a pre-scan. The spotted position markers (Cy3 dye) resulted in an intense signal and revealed good spot quality, at least for the dye spots.

3.1.2 Preparation of the internal standard *rpos*

The *rpoS* gene of *E. coli* encodes the stationary phase sigma factor and was used as internal standard in the microarray experiments. Linearised plasmid containing the *rpoS* gene was used as template for *in vitro* transcription to generate *rpoS*-RNA (see 2.7.4). PCR product of *rpoS* (kindly provided by Dr. A. Zaigler, formerly University of Frankfurt) was printed eight

fold and evenly distributed onto each of the arrays. The average intensity ratio of the *rpoS* spots per slide was used for the normalisation of the obtained data (see 2.7.7).

3.1.3 Target generation: *T. tenax* cultures, preparation of total RNA and cDNA synthesis

Autotrophic and heterotrophic *T. tenax* cultures (100 l) were grown anaerobically at 86°C as described previously (see 2.3). Cells were quickly cooled down to 4°C and harvested via centrifugation at different growth phases (30 l each): Early exponential (1-2 x 10^7 cells/ml), exponential (6-7 x 10^7 cells/ml) and early stationary growth phase (1 x 10^8 cells/ml). Total RNA was isolated of the independently grown *T. tenax* cultures as described previously (see 2.6.2) and immediately after cell lysis, 40 ng of *ropS* RNA were added to each sample. For the control experiment (see 3.1.4.1 and fig. 3.4) two independent autotrophic cultures were grown and harvested in exponential growth phase. The concentration of the RNA samples was determined photometrically at 260 nm and the integrity was checked by agarose gel electrophoresis (see fig. 3.2) and Northern blotting.

RNA isolated from cells harvested in early stationary growth phase revealed problems concerning quality (integrity) and amount of obtained RNA. Furthermore, hybridisation experiments performed with RNA isolated from cells harvested in early exponential growth phase, showed a transcription pattern, which obviously was influenced by the initially added yeast extract to the growth medium (see 2.3; data not shown). Additionally, experiments comparing autotrophic growth on CO_2 and heterotrophic growth on glucose with glycerol grown *T. tenax* cultures failed due to poor growth of *T. tenax* on glycerol.

Therefore, the following data and discussed results refer to RNA, which was isolated of *T. tenax* cells grown auto- and heterotophically and harvested in exponential growth phase.

The RNA samples intended for cDNA synthesis were checked for residual genomic DNA contamination using 1 µl of the preparation as template in a PCR reaction (see 2.6.2). In case of contamination with genomic DNA, the sample was removed and the preparation repeated.

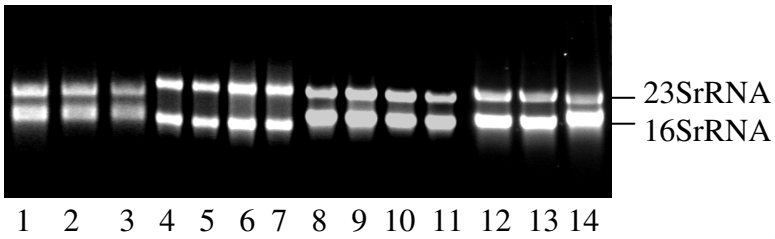


Fig. 3.2 **Agarose gel electrophoresis of total RNA preparations.** The RNA was derived from seven independent heterotrophic (lanes 1-7) and seven independent autotrophic (lane 8-14) cultures, harvested in exponential growth phase. 1 µl RNA sample: Lane 1: 1.7 µg, 2: 1.3 µg, 3: 1.1 µg, 4: 1.8 µg, 5: 1.7 µg, 6: 2.5 µg, 7: 2.1 µg, 8: 3.1 µg, 9: 3.1 µg, 10: 4.0 µg, 11: 4.1 µg, 12: 2.9 µg, 13: 3.5 µg, 14: 2.9 µg.

For the reverse transcriptase (RT) reaction 15 µg of the total RNA were used (see 2.7.5), yielding Cy3 or Cy5 labelled cDNA (target) for the hybridisation to the array.

3.1.4 Hybridisation experiments

Hybridisation was performed in a hybridisation chamber over night at 60°C. After washing, the slides were dried and scanned by using a GenePix 4000a scanner measuring the Cy3 and Cy5 fluorescence intensities at 532 and 635 nm, respectively (see fig. 3.3).

3.1.5 Data processing and analysis

Image processing and spot analysis, including local background correction of the spot surrounding, was performed by using GenePixPro 3.0 software as described previously (see 2.7.7). The average Cy3 and Cy5 intensities of the buffer spots (negative controls) per slide, revealed no high background intensities (< 400 intensity units).

The obtained intensities for the 111 arrayed *T. tenax* sequences at 532 (Cy3) and 635 nm (Cy5) were normalised using the method of internal standardisation by the average intensity ratio of the *rpoS* gene. Further data processing and analysis was performed as described previously (see 2.7.7) using GenePixPro 3.0, GeneSpring software and Microsoft Excel.

Fig. 3.4 (A) shows the results of the control experiment comparing RNA samples derived from two cultures of *T. tenax* grown independently under the same conditions (autotrophic growth). The two RNA samples were differentially labelled, pooled and hybridised to the same slide. Opposing an intensity scatter plot of hybridisation experiment 4 (cDNA derived from glucose- compared to CO_2-grown cells), that clearly shows an effect on gene expression due to the different carbon sources (see fig. 3.5 B).

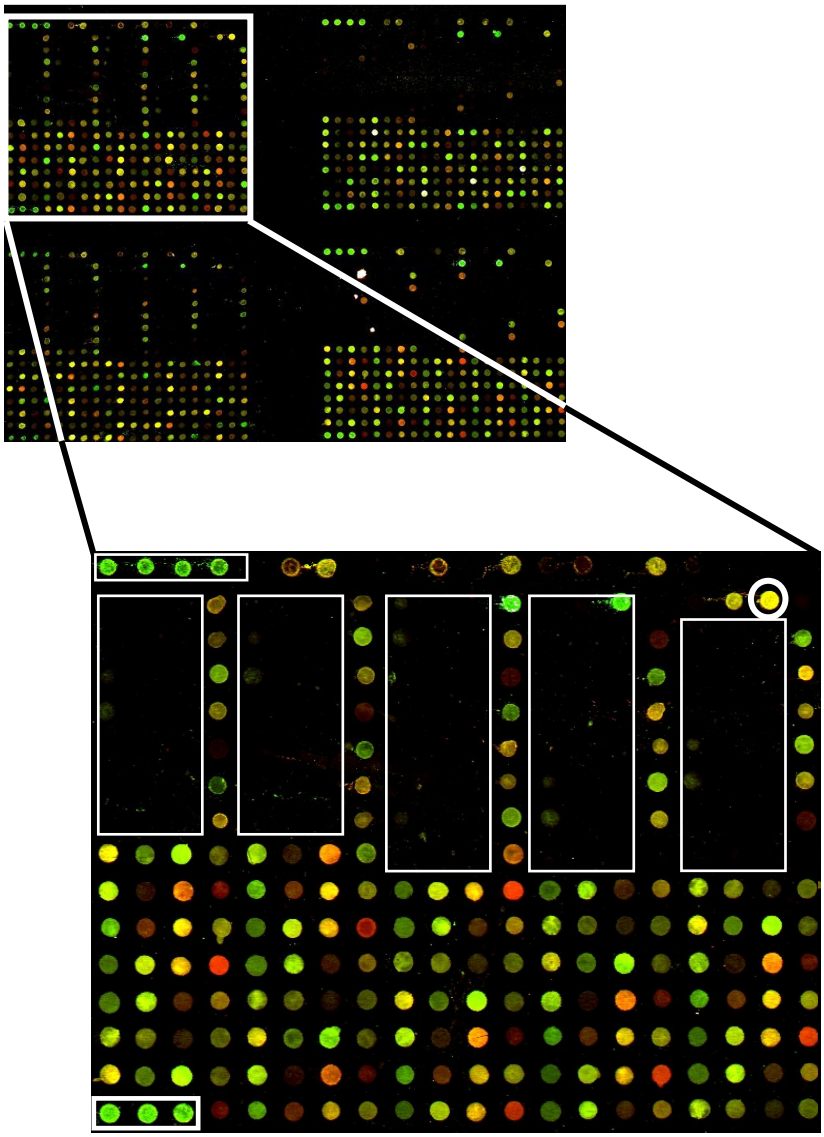


Fig. 3.3 **Picture of a *T. tenax* microarray.** Spotted Cy3 position marker (boxed) and r*poS* spot (encircled) are shown. The big boxes border buffer spots on the array. By an evenly distributed arrangement of the six spots per ORF on the array, occurring local bounded intense background signals did not prohibit evaluable data in most cases.

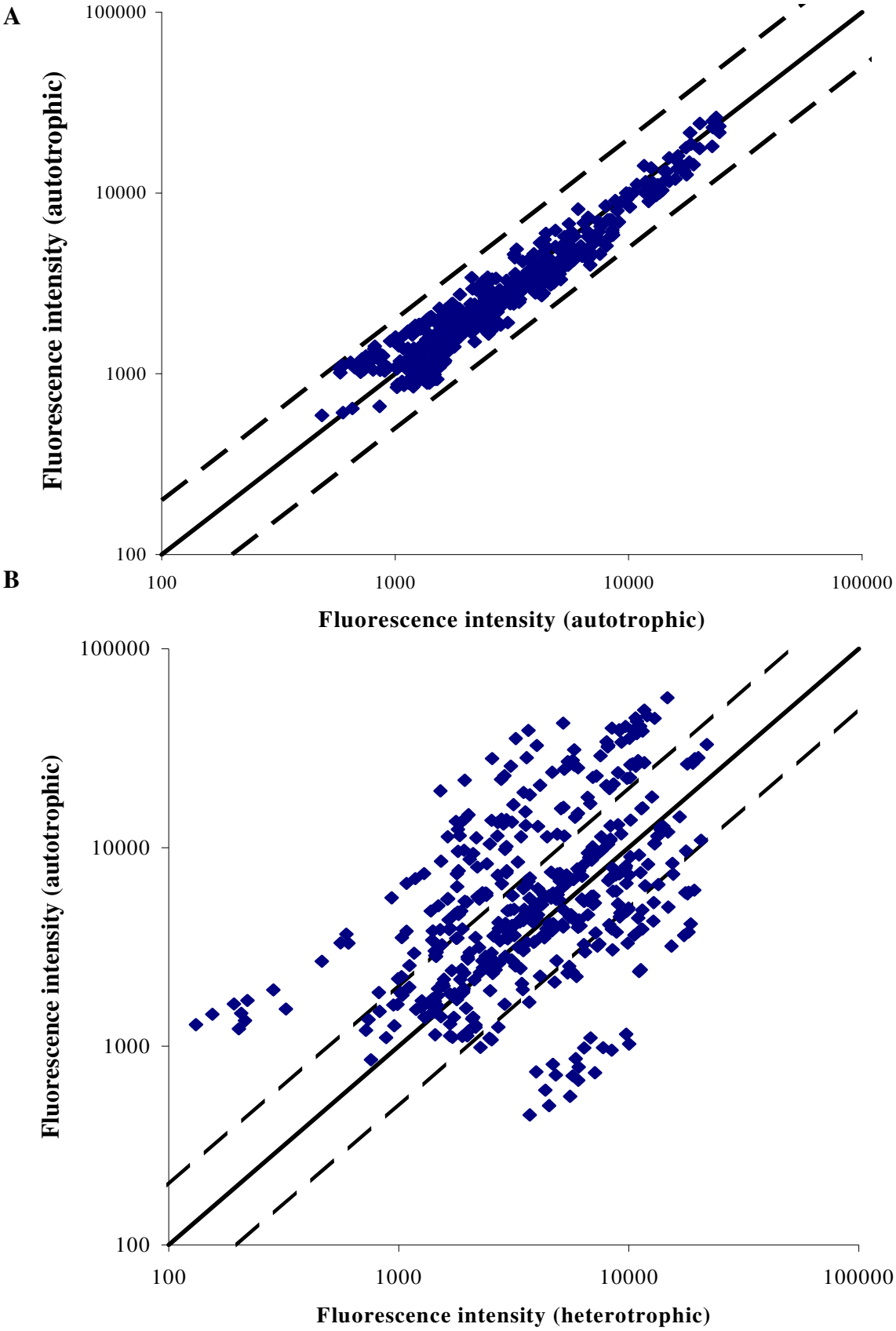


Fig. 3.4 **Quality control of the microarray analyses. A)** Intensity scatter plots of the Cy5 versus the Cy3 intensities of the control experiment and **B)** of experiment 4, comparing an autotrophic versus a glucose-grown culture. The diagonal: no differential regulation, and the upper and lower diagonal: a twofold induction under autotrophic and a twofold induction under heterotophic growth conditions, respectively.

The results of the control experiment show that all signals are very close to the diagonal and therefore reveal no differential gene expression. In case of experiment 4 a lot of spots are located beyond the upper and lower diagonal indicating a differential expression more than twofold.

t Test analysis was performed using GeneSpring software and Microsoft Excel. ORFs with a statistical significance of $P < 0.05$ were counted as significantly expressed and those ORFs that showed expression changes about twofold were accounted as differentially expressed genes (see tab. 3.1).
In table 3.1 the mean intensity ratios of all sequences are given as a $\log_2$ value and each $\log_2$ value is calculated from averaged five hybridisation experiments performed with cDNA derived from 14 independent cultures of *T. tenax*: Seven grown under autotrophic conditions and seven grown heterotrophically on glucose (see 3.1.3). The results of the single hybridisation experiments (1-7) are shown in table A2.1 (see appendix) and the measured Cy5 and Cy3 intensities for each spot are listed in tables A2.3 and A2.2 (control; see appendix).
A total of 90 of the 105 ORFs (111 sequences, respectively) turned out to be expressed in *T. tenax* under the chosen growth conditions glucose and CO_2, respectively, and were used for further analysis. For 15 ORFs no signal could be detected suggesting that these ORFs are not expressed under the chosen growth conditions (NF, tab.1). A total of 60 candidates of the 90 expressed ORFs, show a statistically significant expression ($P < 0.05$), 28 of which resulted to be differentially expressed. Altogether there are 14 genes induced more than twofold in response to the offered carbon source glucose and 14 genes are up-regulated under growth on CO_2 (see tab. 3.1 and fig. 3.5). The regulated genes are distributed among the different CCM pathways as follows: Six of 16 employed EMP genes, only two of eleven genes involved in the ED pathway, 15 of 31 CAC genes, two ferredoxin(Fd)-dependent oxidoreductases as well as three of the four transporter are significantly differential expressed.

Tab.3.1 **Expression ratios of the CCM genes of *T. tenax* grown autotrophically (on CO_2) and heterotrophically (on glucose).** After normalisation of the data, the average of the six spots was calculated, then, the ratios were log transformed (log_2(A/H)) and finally, the log-transformed ratios from the seven slides (mean of five) were averaged. The numbering of the ORFs in the last column corresponds to the numbers indicated in figure 3.5.

No. ORF	orfID (TTX_)	Gene	Gene product	EC no.	COG no.	Comparison autotrophic vs heterotrophic growth		No. Fig. 3.5
						Mean intensity ratio (log_2 +/-SD)[a,c]	Change expression	
Reversible Embden-Meyerhof-Parnas (EMP) pathway								
1	0059	*hp*	Hypothetical protein			0.45 +/-0.4[b]	1.4	
2	0060	*hxk*	ATP-dependent hexokinase (HK)	2.7.1.1	1940	0.47 +/-0.9	1.4	1
3	1762	*hp*	Conserved hypothetical protein /Archaeal-type FBPase, N-terminus	3.1.3.11	0483	0.44 +/-0.8	1.4	4
4	1762	*hp*	Conserved hypothetical protein /Archaeal-type FBPase, C-terminus	3.1.3.11	0483	0.66 +/-1.0	1.6	
5	0980	*pgi*	Glucose-6-phosphate isomerase (PGI)	5.3.1.9	0166	0.46 +/-0.8	1.4	2
6	1277	*pfp*	PP_i-dependent phosphofructokinase (PPi-PFK)	2.7.1.90	0205	-3.04 +/-0.9	8.2	3
7	1278	*fba*	Fructose-bisphosphate aldolase (FBPA)	4.1.2.13	1830	-3.2 +/-0.6	9.2	5
8	0494	*tpi*	Triosephosphate isomerase (TIM)	5.3.1.1	0149	-0.46 +/-0.2	1.4	6
9	1518	*gldA*	Glycerol-1-phosphate dehydrogenase (GLPDH)	1.1.1.261	0371	-0.34 +/-0.7	1.3	7
10	1169	*gapN*	Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN)	1.2.1.9	1012	-0.25 +/-0.4[b]	1.2	10
11	1534	*gap*	Phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	1.2.1.13	0057	1.94 +/-0.6	3.8	8
12	2037	*gor*	Ferredoxin-dependent glyceraldehyde-3-phosphate oxidoreductase (GAPOR)	1.2.7.6	2414	-1.96 +/-0.1	3.9	11
13	1535	*pgk*	3-phosphoglycerate kinase (PGK)	2.7.2.3	0126	2.42 +/-0.5	5.4	9
14	2061	*gpmA*	Phosphoglycerate mutase (PGAM)	5.4.2.1	3635	-0.05 +/-0.3	1.0	12
15	1889	*eno*	Enolase (ENO)	4.2.1.11	0148	0.25 +/-0.3	1.2	13
16	1891	*pyk*	Pyruvate kinase (PK)	2.7.1.40	0469	-0.35 +/-0.4[b]	1.3	14
17	0910	*pps*	Phosphoenolpyruvate synthetase (PEPS)	2.7.9.2	0574	3.52 +/-0.9	11.5	15
18	0683	*ppdk*	Pyruvate,phosphate dikinase (PPDK)	2.7.9.1	0574	-0.69 +/-0.5	1.6	16

Catabolic Entner-Doudoroff (ED) pathway								
19	0329	*gdh*	Glucose dehydrogenase (GDH)	1.1.1.47	1063	0.19 +/-0.5	1.1	17
20	1156	*gad*	Gluconate dehydratase (GAD)	4.2.1.39	4948	-0.60 +/-0. 6	1.5	18
21	1156a	*kdgA*	2-keto-3-deoxy-(phospho)gluconate aldolase (KD(P)GA)	4.1.2.-	0329	-0.27 +/-0.5 [b]	1.2	20
22	1157	*kdgK*	2-keto-3-deoxy-gluconate kinase (KDGK)	2.7.1.45	0524	-0.14 +/-0.5 [b]	1.1	19
23	1396	*aor-1*	Fd-dependent aldehyde oxidoreductase (AOR1), candidate 1, C-terminus	1.2.7.-	2414	NF		21
24	1396	*aor-1*	Fd-dependent aldehyde oxidoreductase, candidate 1 (AOR1), N-terminus	1.2.7.-	2414	-0.33 +/-0.4	1.3	
25	1294	*aor-2*	Fd-dependent aldehyde oxidoreductase, candidate 2 (AOR2)	1.2.7.-	2414	NF		21
26	1768	*aor-3*	Fd-dependent aldehyde oxidoreductase, candidate 3 (AOR3) , N-terminus	1.2.7.-	2414	-0.36 +/-1.9	1.3	21
27	1768	*aor-3*	Fd-dependent aldehyde oxidoreductase, candidate 3 (AOR3) , C-terminus	1.2.7.-	2414	-0.04 +/-2.0 [b]	1.0	
28	1787	*aldh-1*	Aldehyde dehydrogenase, candidate 1 (ALDH1)	1.2.1.-	1012	-0.45 +/-0.5	1.4	22
29	1101	*aldh-2*	Aldehyde dehydrogenase, candidate 2 (ALDH2)	1.2.1.-	1012	NF		22
30	0788	*garK*	Glycerate kinase (GK)	2.7.1.31	2379	1.14 +/-0.7	2.2	23
31	0789	*edd / ilvD*	6-phoshogluconate dehydratase (EDD) / Dihydroxy-acid dehydratase (DHAD)	4.2.1.12/ 4.2.1. 9	0129	1.17 +/-0.4	2.3	
Reversible citric acid cycle (CAC)								
37	0497	*gltA*-1	Citrate synthase 1 (CS1)	2.3.3.1	0372	0.17 +/-2.1	1.1	25
38	1513	*gltA*-2	Citrate synthase 2 (CS2)	2.3.3.1	0372	-1.23 +/-0.2	2.4	26
39	1436	*act (citF)*	Citrate lyase, α-subunit (CL)	4.1.3.6	1804	-0.47 +/-1.1	1.4	27
40	1435	*citE*	Citrate lyase, β-subunit (CL)	4.1.3.6	2301	-0.67 +/-0.8	1.6	27
41	1437	*acs*	Citrate lyase, γ-subunit (CL)	6.2.1.1	0365	-0.06 +/-0.8 [b]	1.0	27
42	0493	*acn*	Aconitase (ACN)	4.2.1.3	1048	-1.3 +/-0.4	2.5	28
43	1489	*idhA*	Isocitrate dehydrogenase (IDH)	1.1.1.42	0538	-1.4 +/-1.1	2.6	29
44	0334	*lpd-1*	Oxoglutarate dehydrogenase, candidate 1	1.8.1.4	1249	NF		30
45	0406	*lpd-2*	Oxoglutarate dehydrogenase, candidate 2	1.8.1.4	1249	NF		30
46	2034	*oorA*	Fd-dependent 2-oxoglutarate oxidoreductase, candidate 1, α-subunit (OOR)	1.2.7.-	0674	-0.82 +/-0.4	1.8	31

47	2033	*oorB*	Fd-dependent 2-oxoglutarate oxidoreductase, candidate 1, β-subunit (OOR)	1.2.7.-	1013	-0.87 +/-0.3	1.8	31
48	2036	*oorC*	Fd-dependent 2-oxoglutarate oxidoreductase, candidate 1, γ-subunit (OOR)	1.2.7.-	1014	-1.06 +/-0.5	2.1	31
49	2035	*oorD*	Fd-dependent 2-oxoglutarate oxidoreductase, candidate 1, δ-subunit (OOR)	1.2.7.-	1144	-1.08 +/-0.5	2.1	31
50	0209	*oorA*	Fd-dependent 2-oxoglutarate oxidoreductase, candidate 2, α-subunit (OOR)	1.2.7.-	0674	2.24 +/-0.7	4.7	31
51	0210	*oorB*	Fd-dependent 2-oxoglutarate oxidoreductase, candidate 2, β-subunit (OOR)	1.2.7.-	1013	2.33 +/-0.7	5.0	31
52	0208	*oorCD*	Fd-dependent 2-oxoglutarate oxidoreductase, candidate 2, γ/δ-subunit (OOR)	1.2.7.-	1014	1.82 +/-0.3	3.5	31
53	0922	*sucD*	Succinyl-CoA synthetase, α-subunit	6.2.1.5	0074	0.16 +/-0.5 [b]	1.1	32
54	0921	*sucC*	Succinyl-CoA synthetase, β-subunit	6.2.1.5	0045	-0.23 +/-0.5 [b]	1.2	32
55	0864	*sdhA* (*frdA*)	Succinate dehydrogenase (fumarate reductase), candidate 1, α-subunit	1.3.99.1	1053	-2.9 +/-0.7	7.5	33
56	0863	*sdhB* (*frdB*)	Succinate dehydrogenase (fumarate reductase), candidate 1, β-subunit	1.3.99.1	0479	-2.23 +/-0.7	4.7	33
57	0862	*sdhC* (*frdC*)	Succinate dehydrogenase (fumarate reductase), candidate 1, γ -subunit	1.3.99.1	1053	-1.57 +/-0.6	3.0	33
58	0861	*sdhD* (*frdD*)	Succinate dehydrogenase (fumarate reductase), candidate 1, δ -subunit	1.3.99.1	-	-1.69 +/-0.7	3.2	33
59	1104	*frdA*	Fumarate reductase (Succinate dehydrogenase), candidate 2, α-subunit	1.3.99.1	1053	3.64 +/-0.9	12.5	34
60	1105	*frdB*	Fumarate reductase (Succinate dehydrogenase), candidate 2, β -subunit	1.3.99.1	0479	2.04 +/-0.6	4.1	34
61	1106	*adh*	Zn^{2+}-dependent alcohol dehydrogenase class III (ADH)	1.1.1.1	1062	3.4 +/-1.8	10.6	
62	1295	*fumC*	Fumarate hydratase class II	4.2.1.2	0114	-0.63 +/-0.2	1.6	35
63	1765	*fumA/ttdA*	Fumarate hydratase class I / tartate dehydrogenase, α-subunit	4.2.1.2 / 4.2.1.32	1951	-0.42 +/-0.3	1.3	35
64	1764	*fumB/ttdB*	Fumarate hydratase class I / tartate dehydrogenase, β -subunit	4.2.1.2 / 4.2.1.32	1838	NF		35
65	1427	*mdh*	Malate dehydrogenase (MDH)	1.1.1.37	0039	-0.74 +/-0.2	1.7	36
66	1514	*mae*	Malic enzyme (MAE)	1.1.1.38	0281	-0.04 +/-0.8 [b]	1.0	37

67	1316	*glcB*	Malate synthase (MS), N-terminus	2.3.3.9	2225	NF		
68	1316	*glcB*	Malate synthase (MS), C-terminus	2.3.3.9	2225	NF		
Pentose metabolism								
32	2039	*rpiA*	Ribosephosphate isomerase	5.3.1.6	0120	0.64 +/-0.1	1.6	38
33	1754	*tktA*	Transketolase, N-terminal section	2.2.1.1	3959	0.46 +/-0.9	1.4	40
34	1753	*tktB*	Transketolase, C-terminal section	2.2.1.1	3958	0.28 +/-0.9 [b]	1.2	40
35	0613	*deoC*	Deoxyribose-phosphate aldolase (DERA)	4.1.2.4	0274	0.03 +/-0.3 [b]	1.0	39
36	1882	*rbsK*	Ribokinase	2.7.1.15	0524	0.33 +/-0.6 [b]	1.3	41
Glycogen metabolism								
69	2058	*pgm/manB*	Phosphoglucomutase / phosphomannomutase	5.4.2.2 / 5.4.2.8	1109 /	-0.47 +/-0.6	1.4	44
70	0995	*snt*	Sugar nucleotidyl transferase, candidate 1	2.7.7.-	1208	-0.06 +/-0.4 [b]	1.0	
71	1336	*rfbA*	Sugar nucleotidyl transferase, candidate 2 dTDP-D-glucose thymidyl transferase	2.7.7.24	1209	NF		
72	1335	*rfbB*	dTDP-glucose-4,6- dehydratase	4.2.1.46	1088	NF		
73	0596	*snt-5*	Sugar nucleotidyl transferase, candidate 3	2.7.7.-	1208	-0.21 +/-0.4 [b]	1.2	
74	1400	*glgA*	Glycogen (starch) synthase (GLGA)	2.4.1.11	0297	0.21 +/-0.03 [b]	1.2	45
75	1397	*glgP*	Glycogen phosphorylase (GLGP)	2.4.1.1	0058	-0.22 +/-0.4 [b]	1.2	43
76	1399	*amyA*	α-amylase	3.2.1.1	1449	0.25 +/-0.2 [b]	1.2	42
77	1158	*gaa*	Glucan 1,4- α-glucosidase (Glucoamylase)	3.2.1.3	3387	0.28 +/-0.3	1.2	42
78	1745	*malZ*	α-glucosidase (Maltase)	3.2.1.20	1501	0.01 +/-0.5 [b]	1.0	
Trehalose metabolism								
79	1304	*tpsp*	Trehalose-6-phosphate synthase/phosphatase (TPSP)	2.4.1.15 / 3.1.3.12	0380	NF		46
80	1304a	*msc*	Small conductance mechanosensitive channel (MscTTX)			0.39 +/-0.35 [b]	1.3	
81	1305	*gt*	Glycosyl transferase (GT)	2.4.1.-	0438	NF		47
82	0218	*hp*	Hypothetical protein			NF		
83	0217	*treT*	Putative glycosyl-transferring trehalose synthase (TreT)			0.29 +/-0.83 [b]	1.2	
Carbon monoxide dehydrogenase								
84	0328	*coxM/cutM*	Carbon monoxide dehydrogenase, medium subunit	1.2.99.2	1319	-0.2 +/-0.6	1.2	
85	0327	*coxS/cutS*	Carbon monoxide dehydrogenase, small subunit	1.2.99.2	2080	0.05 +/-0.3 [b]	1.0	

86	0326	*coxL/cutL*	Carbon monoxide dehydrogenase, large subunit	1.2.99.2	1529	0.03 +/-0.3 [b]	1.0
Ferredoxin (Fd)-dependent oxidoreductases (OR)							
87	1758	*oorCA*	Pyruvate:Fd OR, 2-oxoacid Fd:OR, candidate 1, γ-α-subunit	1.2.7.-	0674	-0.92 +/-1.2	1.9
88	1757	*oorB*	Pyruvate:Fd OR, 2-oxoacid Fd:OR, candidate 1, β-subunit	1.2.7.-	1013	0.04 +/-1.0 [b]	1.0
89	1455	*oorCA*	Pyruvate:Fd OR, 2-oxoacid Fd:OR, candidate 2, γ-α-subunit, N-terminus	1.2.7.-	0674	NF	
90	1455	*oorCA*	Pyruvate:Fd OR, 2-oxoacid Fd:OR, candidate 2, γ-α-subunit, C-terminus	1.2.7.-	0674	NF	
91	1454	*oorB*	Pyruvate:Fd OR, 2-oxoacid Fd:OR, candidate 2, β-subunit	1.2.7.-	1013	NF	
92	1785	*oorA*	Pyruvate:Fd OR, 2-oxoacid Fd:OR, candidate 3, α-subunit	1.2.7.-	0674	-1.57 +/-0.3	3.0
93	1786	*oorB*	Pyruvate:Fd OR, 2-oxoacid Fd:OR, candidate 3, β-subunit	1.2.7.-	1013	-1.21 +/-0.7	2.3
94	0712	*iorA*	Indolepyruvate Fd:OR (IOR), α-subunit	1.2.7.8	4231	-1.11 +/-1.9 [b]	2.2
95	0713	*iorB*	Indolepyruvate Fd:OR (IOR), β-subunit	1.2.7.8	1014	-0.93 +/-2.9 [b]	1.9
Polyphosphate metabolism							
96*	0388	*ppa*	Inorganic pyrophosphatase	3.6.1.1	0221	1.65 +/-0.3	3.1
97*	1550	*hppa*	H^+ translocating pyrophosphatase synthase, N-terminus	3.6.1.1	3808	-0.8 +/-0.7	1.7
98*	1550	*hppa*	H^+ translocating pyrophosphatase synthase, C-terminus	3.6.1.1	3808	-0.7 +/-0.7	1.6
99*	0826		Exopolyphosphatase-related protein			NF	
Protein phosphorylation							
100*	1664		Serine/threonine protein kinase		0478	NF	
101*	1913		Serine/threonine protein kinase, C-terminus	2.7.1.37	0515	NF	
102*	0682	*serB*	Phosphoserine phosphatase	3.1.3.3	0560	NF	
103*	0684	*ppa*	Serine/threonine specific protein phosphatase	3.1.3.16	0639	NF	
Transport							
104*	0482		ABC-type branched-chain aa binding protein		0683	-0.14 +/-1.6	1.1
105*	1140		ABC-type branched-chain aa transport protein		0683	1.71 +/-0.5	3.3
106*	0155		oligopeptide binding protein		3889	1.74 +/-0.7	3.3

107*	0083		ABC-type dipeptide transporter, substrate binding protein	6747	1.86 +/-1.4	3.6
Transcription						
108*	2085	*tfb2*	Transcription initiation factor TFIIB (TFB2)	1405	0.92 +/-0.8	1.9
109*	1484	*tfb1*	Transcription initiation factor TFIIB (TFB1)	1405	-0.63 +/- 0.7	1.6
110*	0178	*tbp*	TATA box binding protein (TBP)	2101	1.2 +/-0.3	2.3
111*	0985	*napF*	Ferredoxin	1145	0.9 +/-0.3	1.9

[a] The mean intensity ratio is given as a $\log_2$ value. Each $\log_2$ value follows from averaged five hybridisation experiments; the standard deviation (SD, +/-) is given
For all ORFs expression is statistically significant (*P* value < 0.05) unless otherwise indicated
[b] *p* value > 0.05
[c] NF – signal not found
* Designated "non-CCM" ORFs, excluded from further discussion (for detailed results see Tab. A2.1 and A2.3, appendix)

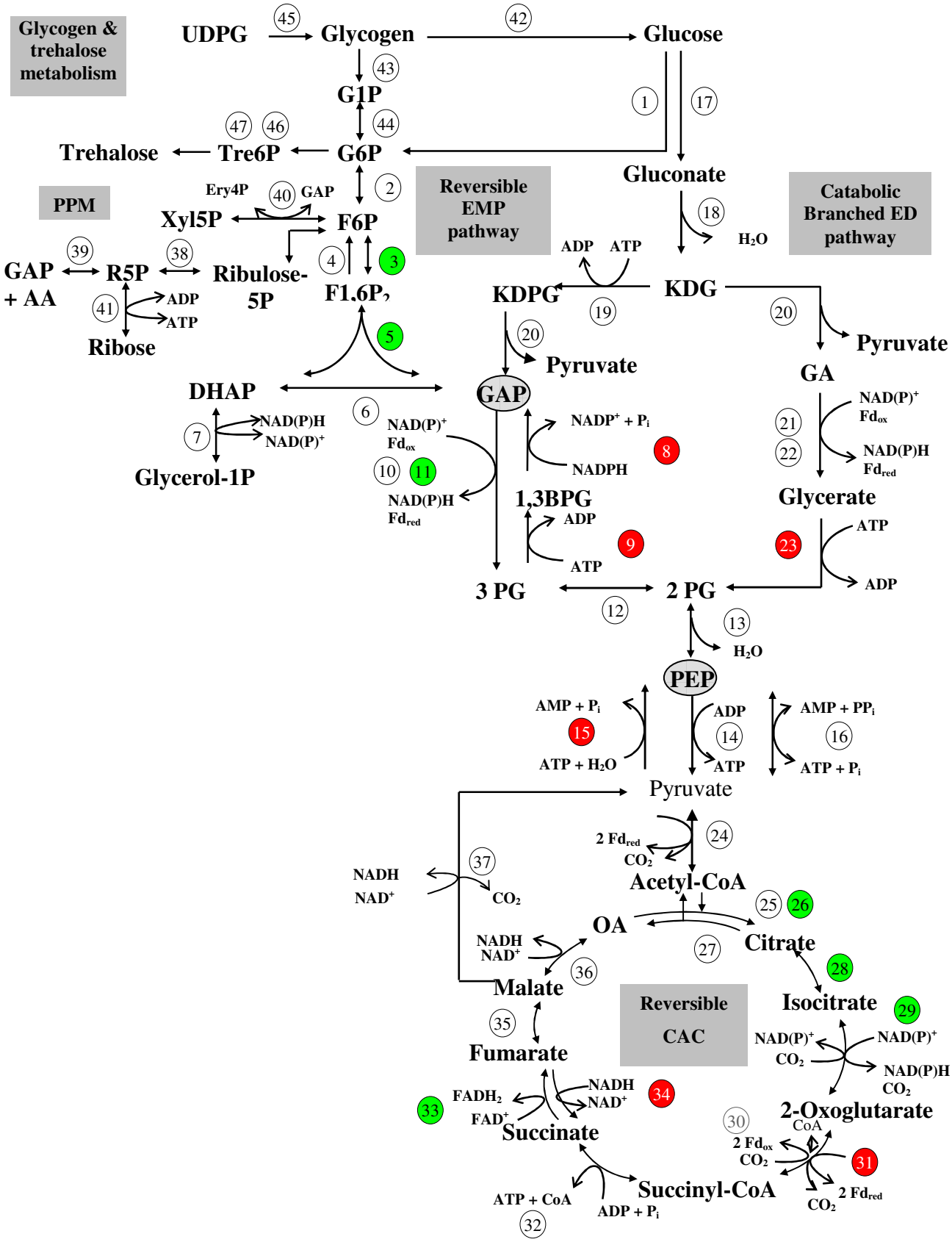


Fig. 3.5 **Overview of the CCM of *T. tenax*.** Encircled numbers correspond to table 3.1 (last column). Gene regulation is indicated by red: induction of gene expression under autotrophic growth conditions, and green coloring: induction in glucose-grown cells.

3.1.5.1 The reversible Embden-Meyerhof-Parnas (EMP) pathway

The reversible EMP pathway of *T. tenax* represents the main route for glucose catabolism and the pathway is also utilised for the phosphorolytic degradation of the storage compound glycogen. Additionally, the complete set of genes involved in the anabolic direction (gluconeogenesis) is present (Siebers *et al.*, 2004).

A total of six genes of the 16 genes involved in the modified *T. tenax* EMP pathway show a differential expression more than twofold in response to the offered carbon source glucose or CO_2. Gene expression of the reversible PP_i-dependent phosphofructokinase (PP_i-PFK, *pfp*; TTX_1277; fig. 3.5 no.3) and fructose-1,6-bisphosphate aldolase (FBPA, *fba*; TTX_1278; fig. 3.5 no. 5) as well as the catabolic ferredoxin-dependent glyceraldehyde-3-phosphate oxidoreductase (GAPOR, *gor*; TTX_2037; fig. 3.5 no. 11) are significantly induced in response to glucose (repressed under autotrophic growth, respectively). In contrast, the anabolic phosphoenolpyruvate synthetase (PEPS, *pps*; TTX_0910; fig. 3.5 no.15), phosphoglycerate kinase (PGK, *pgk* TTX_1535; fig. 3.5 no. 9) and the classical $NADP^+$-dependent GAP dehydrogenase (GAPDH, *gap*; TTX_1534; fig. 3.5 no. 8) are up-regulated in response to CO_2.

Most remaining genes engaged in the EMP pathway of *T. tenax* show no regulation on transcript level in response to the carbon source glucose or CO_2: ATP-dependent hexokinase (HK, *hxk*; TTX_0060; fig. 3.5 no. 1), glucose-6-phosphate isomerase (PGI, *pgi*; TTX_0980; fig. 3.5 no. 2), archaeal type IV fructose-1.6-bisphosphatase (FBP, *fbp;* TTX_1762; fig. 3.5 no. 4), triosephosphate isomerase (TIM, *tpi*; TTX_0494; fig. 3.5 no. 6), glycerol-1-phosphate dehydrogenase (GLPDH, *gldA*; TTX_1518; fig. 3.5 no. 7), phosphoglycerate mutase (PGAM, *gpmA*; TTX_2061; fig. 3.5 no. 12), enolase (ENO, *eno*; TTX_1889; fig. 3.5 no. 13) and pyruvate, phosphate dikinase (PPDK, *ppdk*; TTX_0683; fig. 3.5 no. 16). Statistically not significant signals ($P > 0.05$) were detected for the ORFs TTX_1169 and TTX_1891 coding for the non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN, *gapN*; fig. 3.5 no. 10) and the pyruvate kinase (PK, *pyk*; TTX_1891; fig. 3.5 no. 14) of *T. tenax*.

3.1.5.2 The catabolic, branched Entner-Doudoroff (ED) pathway

For *T. tenax*, the so-called branched ED pathway has been described (Ahmed *et al.*, 2005). This catabolic pathway reveals an alternative route for glucose degradation to the EMP

pathway, and in addition, the pathway is supposed to be involved in the hydrolytic degradation of glycogen in *T. tenax.*

For most of the ORFs involved in the branched ED pathway no change in transcript amount was observed in response to the carbon source. Only the glycerate kinase (GK, *garK*; TTX_0788; fig. 3.5 no. 23), key enzyme of the non-phosphorylative ED branch, and dihydroxy-acid dehydratase (DHAD, *ilvD*; TTX_0789), which are supposed to form an operon, show a significant up-regulation in response to autotrophic growth conditions. The ORF encoding glucose dehydrogenase (GDH, *gdh*; TTX_0329; fig. 3.5 no. 17) and TTX_1787 encoding aldehyde dehydrogenase candidate 1 (ALDH1, *aldh-1*; fig. 3.5 no. 22) are significantly expressed, but show no regulation depending on the growth conditions (CO_2 / glucose). For the KD(P)G aldolase (KD(P)GA, *kdgA*; TTX_1156a; fig. 3.5 no. 20), KDG kinase (KDGK, *kdgK*; TTX_1157; fig. 3.5 no. 19) and two candidates of ferredoxin(Fd)-dependent aldehyde oxidoreductase (AOR 2/3, *aor-2/3*; TTX_1294 and TTX_1768; fig. 3.5 no. 21) no statistically significant signal ($P > 0.05$) was detected. Anyhow, no tendency for a differential expression appear. For two ED genes encoding another candidate for a ferredoxin(Fd)-dependent aldehyde oxidoreductase (AOR 1, *aor-1*; TTX_1396; fig. 3.5 no. 21) and candidate 2 for an aldehyde dehydrogenase (ALDH2, *aldh-2*; TTX_1101; fig. 3.5 no. 22) no signal was detected at all.

3.1.5.3 The reversible citric acid cycle (CAC)

In *T. tenax* the reversible CAC plays a major role in the complete oxidation of organic compounds to CO_2 (oxidative cycle; Selig and Schönheit, 1994) as well as is supposed to function in CO_2-fixation (reductive cycle; Siebers *et al.*, 2004). Most of the CAC enzymes catalyse easily reversible reactions and as is generally known, only the enzyme couples citrate synthase/citrate lyase, 2-oxoglutarate dehydrogenase/2-oxoglutarate oxidoreductase and succinate dehydrogenase/fumarate reductase are supposed to decide about the direction of the pathway. A complete set of engaged CAC enzymes, with the only exception of a 2-oxoglutarate dehydrogenase, were identified in the *T. tenax* genome (see tab. 3.1). For the 2-oxoglutarate dehydrogenase two candidates encoding only the dihydrolipoamide dehydrogenase (E3) component of the multienzyme complex are found in the genome of *T. tenax* (*lpd*-1, TTX_0334 and *lpd*-2, TTX_0406; fig. 3.6 no. 30). No signals could be detected in the microarray analysis.

The transcript levels of the key enzymes of the oxidative direction one of the two citrate synthases (CS-2, *gltA-2*; TTX_1513; fig. 3.5 no. 26), and the suggested candidates encoding the subunits of the succinate dehydrogenase (*sdhD-sdhC-sdhB-sdhA* operon, TTX_0861-0862-0863-0864; fig. 3.6 no. 33) are significantly up-regulated in glucose-grown cells (see tab.3.1). Whereas, the ORF encoding the citrate synthase 1 (CS-1, *gltA-1*; TTX_0497; fig. 3.5 no. 25) is not influenced by growth on glucose or CO_2.

The reductive direction of the CAC is determined by the action of the predicted fumarate reductase (*frdA-frdB*, TTX_1104-1105; fig. 3.5 no. 34) and the encoding ORFs are induced in CO_2-grown cells. However, no change of transcript amount was observed for the predicted citrate lyase (CL, TTX_1436, α-, TTX_1435, β- and TTX_1437 γ-subunit; fig. 3.5 no. 27) under growth on CO_2. But, in addition, a significant higher transcript level is found for one of the two identified candidates coding for 2-oxoglutarate oxidoreductase (OOR, *oorA-oorB-oorCD* operon, TTX_0208-0209-0210; fig. 3.5 no. 31) under autotrophic conditions, which is suggested to operate in both directions in *T. tenax* (Siebers *et al.* 2004). The four subunits of the second candidate, also seem to be organised in an operon (*oorA-oorB-oorC-oorD* operon, TTX_2033-2034-2035-2036; fig. 3.5 no. 31) that is expressed, no differential gene expression could be observed during growth on glucose or CO_2 for the α- and β-subunit, however, γ- and δ-subunit seem to be induced in CO_2-grown cells (see table 3.1).

In Archaea, the oxidation of pyruvate to acetyl-CoA, is catalysed by pyruvate-ferredoxin oxidoreductase (POR; fig. 3.6 24). Due to the high sequence similarity between Fd:oxidoreductases, an unequivocal annotation of the OOR and POR revealed problems. Therefore, the candidates TTX_0208-0209-0210 and TTX_2033-2034-2035-2036, that both consist of four subunits, can either represent POR or OOR.

The aconitase (ACN, *acn*; TTX_0493; fig. 3.5 no. 28) encoding ORF as well as the ORF coding for the isocitrate dehydrogenase (IDH, *icd*; TTX_1489; fig. 3.5 no. 29) catalyse reversible reactions of the CAC and the encoding genes are induced under heterotrophic growth conditions.

All remaining ORFs encoding reversible CAC enzymes (succinyl-CoA synthetase subunits (*sucD-sucC*, TTX_0922-0921; 32), malate dehydrogenase (MDH, *mdh*, TTX_1427; fig. 3.5 no. 36), class II fumarase (FUM, *fumC*; TTX_1295; fig. 3.5 no. 35) show no differential gene expression. TTX_1765 and 1764 show similarity to the subunits of bacterial class I FUM (*fumC*) or to the α- and β-subunit of tartate dehydrogenase (*fumA/t-fumB/t*). However, the encoding ORFs are either not expressed (TTX_1764) or not influenced by the given carbon sources (TTX_1765).

For the ORF TTX_1524 coding for malic enzyme (MAE, *mae*, TTX_1514; fig. 3.5 no. 37), which catalyses the reversible oxidative decarboxylation of malate to pyruvate, whereas no statistically significant signal ($P > 0.05$) was observed. TTX_1316 shows similarity to malate synthase (MS, *glcB*), generally involved in the glyoxylate shunt, but no signal was detected. The glyxoylate shunt is supposed to be generally absent in *T. tenax*, because no homolog of an isocitrate lyase, the second key enzyme of the cycle could be identified in the *T. tenax* genome.

The ORFs TTX_0328-0327-0326 (84-86) show homology to the medium, small and the large subunit of carbon monoxide dehydrogenase, the key enzyme of the reductive acetyl CoA (Wood-Ljungdahl) pathway, an alternative route for CO_2 fixation. However, the encoding ORFs are not expressed (see tab. 3.1).

3.1.5.4 Pentose phosphate metabolism

Unfortunately, in times of the *T. tenax* CCM DNA microarray design the archaeal reversed RuMP pathway via 3-hexulose-6-phosphate isomerase (PHI) and the 3-hexulose-6-phosphate synthase (HPS), responsible for the generation of pentoses was unknown. The following genes involved in the metabolism of pentoses in *T. tenax* are comprised on the microarray: The ribosephosphate isomerase (*rpiA*; TTX_2039; fig. 3.5 no. 38), the transketolase (*tktA*, *tktB*; TTX_1754, TTX_1753; fig. 3.5 no. 40), the ribokinase (rbsK; TTX_1882; fig. 3.5 no. 41) and a homolog of a deoxyribose-phosphate aldolase (DERA, *deoC*; TTX_0613; fig. 3.5 no. 39). The microarray experiments revealed that the expression of none of these genes seems to depend on the carbon sources glucose or CO_2, respectively (see tab. 3.1).

3.1.5.5 Glycogen and trehalose metabolism

For all ORFs involved in the metabolism of the storage compound glycogen, that are contained on the microarray no differential expression more than twofold is observed depending on heterotrophic or autotrophic growth, respectively (see tab. 3.1). TTX_1336 encoding sugar nucleotidyl transferase (*rfbA*) and TTX_1335 coding for dTDP-glucose-4.6-dehydratase (*rfbB*), that are supposed to form an operon, are not expressed at least under the selected growth conditions.

For the ORFs involved in the metabolism of trehalose the results obtained from the microarray study are a little ambiguous. Only for two ORFs encoding a hypothetical protein with similarity to a mechanosenstive channel (TTX_1304a; see 3.3.3) and a putative glycosyl-transferring trehalose synthase (TreT; TTX_0217) signals were detected ($P > 0.05$).
No signals were found for the ORFs TTX_1304 and TTX_1305 encoding trehalose-6-phosphate synthase/phosphatase (TPSP; fig. 3.5 no. 46), putative glycolsyl transferase (GT fig. 3.5 no. 47) and also for the ORF TTX_0218, which is supposed to form an operon with TTX_0217 (4 bp overlap).

3.1.6 Northern Blot analyses

In order to verify the obtained microarray data, expression profiles of six genes were checked by Northern blot analyses, that were performed for the following genes: TTX_0910 (*pps*), TTX_1105 (*frdB*), TTX_1513 (*gltA*-2), TTX_1277 (*pfp*), TTX_2061 (*gpmA*) and TTX_1158 (*gaa*) by using radiolabeled antisense mRNA probes (see 2.6.6.1) and total RNA derived from auto- and heterotrophically grown cells (see 2.6.2).
The templates for *in vitro* transcription and simultaneous [^{32}P]-labelling were generated via PCR amplification (see tab. 2.5). A template size of about 500 bp was chosen, with exception for *frdB* (286 bp) and a 1.5 kb 16S rRNA gene fragment. Due to very low labelling efficiency (6,000-10,000 cpm/µl) of *in vitro* transcribed *gltA*-2 RNA, a new forward primer (fII; see tab. 2.5) was used, resulting in a 869 bp PCR product.
In vitro transcription was performed with 100-200 ng purified template DNA in presence of 50 µCi [α-^{32}P]-CTP (400 Ci/mmol) by T7 RNA polymerase (see 2.6.6.1).
8 µg of total RNA derived from auto- and heterotrophically grown *T. tenax* cells harvested in exponential growth phase, were separated via denaturing formaldehyde agarose gel electrophoresis (see 2.6.4) and immobilised on a membrane via capillary transfer (Northern blot; fig. 3.6 and see 2.6.5).

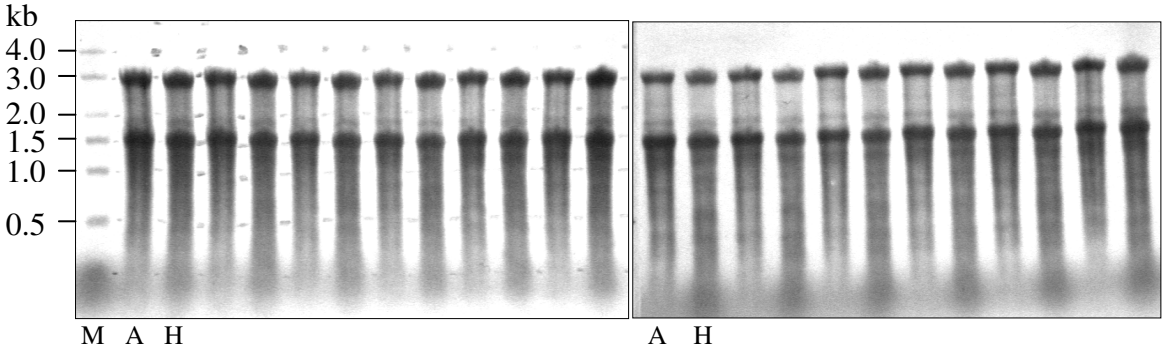


Fig. 3.6 **Methylene stained Northern blots of separated total RNA from auto- (A) and heterotrophically (H) grown *T. tenax* cells.** M: marker, 0.1 µg RNA ladder (high range). Alternating lanes A: 8 µg autotrophic total RNA next to lanes H: 8 µg heterotrophic total RNA

Hybridistaion of the [α-^{32}P]-labelled antisense RNA probes to the immobilised total RNA was performed in UltraHyb hybridisation solution at temperatures between 68°C and 70°C over night, followed by subsequent low- and high stringency washes (see 2.6.6.2). Detection of RNA-RNA hybrids (see 2.6.6.3) was carried out via exposure to a standard X-ray film (see fig. 3.7) for rapid information of experimental success and of adequate exposure times for subsequent phosphor imaging autoradiography performing signal quantitation (see tab. 3.2).

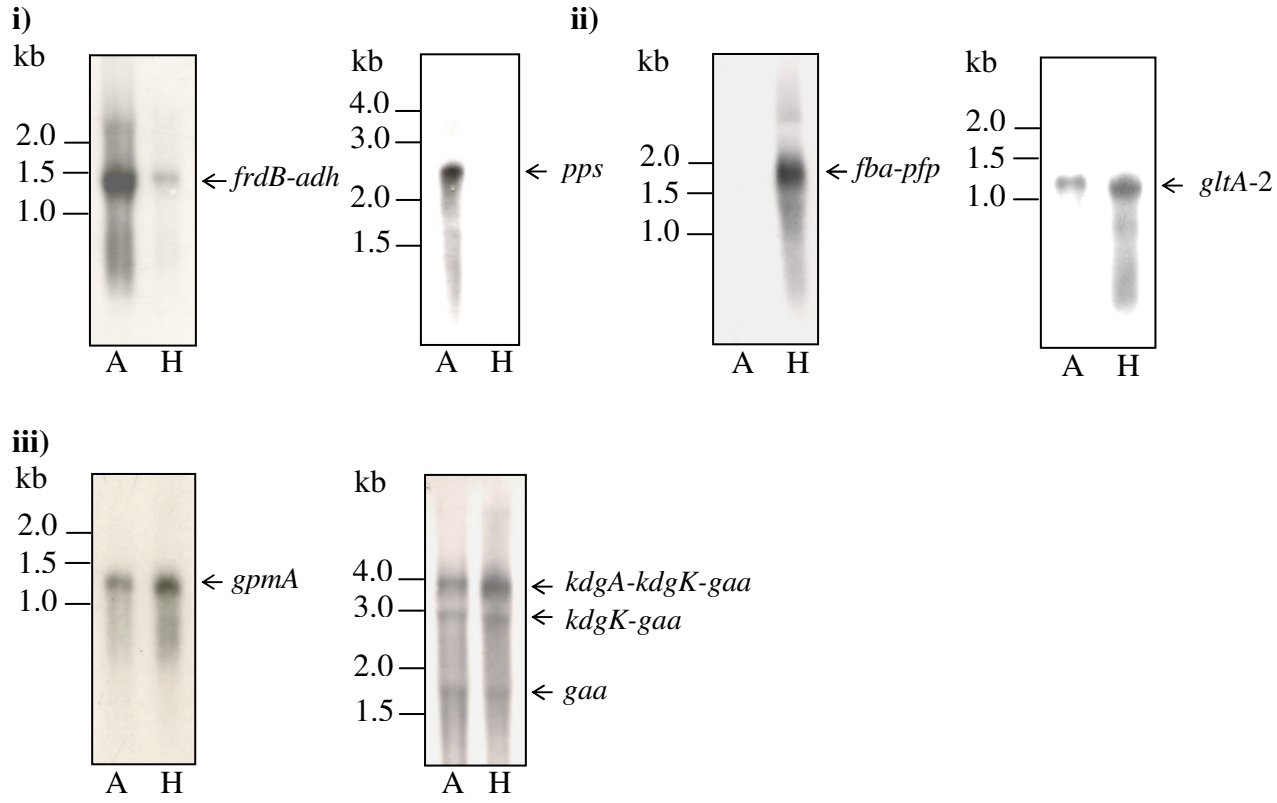


Fig. 3.7 **Northern blot analyses of six selected CCM ORFs. i)** *frdB-adh* and *pps* (induced under autotrophic growth) **ii)** *pfp-fba*, *gltA*-2 (induced under heterotrophic growth

conditions) as well as **iii)** two independently expressed ORFs (*gpmA*, *gaa*).

Tab. 3.2 **Results of the Northern blot analyses compared to intensity change derived from the microarray experiments.** Quantitation of the signals was performed for the *pps*-, *frdB*-, *pfp*-, *gltA-2*-, *gpmA*- and *gaa*- northern blot analyses via phosphor imaging.

orf ID	gene / operon	size (bases)	Intensity change (x fold) Autotrophic vs heterotrophic growth	
			Northern analyses	microarray
TTX_0910	*pps*	2,433	23.3	11.5
TTX_1105	*adh-frdB*	1,092 + 285	7.6	4.1
TTX_1277	*fba-pfp*	1,014 + 792	26.9	8.2
TTX_1513	*gltA-2*	1,233	3.0	2.4
TTX_2061	*gpmA*	1,236	1.2	1.0
TTX_1158	*kdgA-kdgK-gaa*	861 + 933 +1,818	1.4	1.2

16S rRNA served as an internal standard to ensure equal amounts of total autotrophic and heterotrophic RNA, respectively. For all genes, microarray data were confirmed considering ratio deviations (see fig. 3.8 and tab. 3.2).

3.2 Functional analysis of the leucine-responsive regulator protein (LrP1) of *T. tenax*

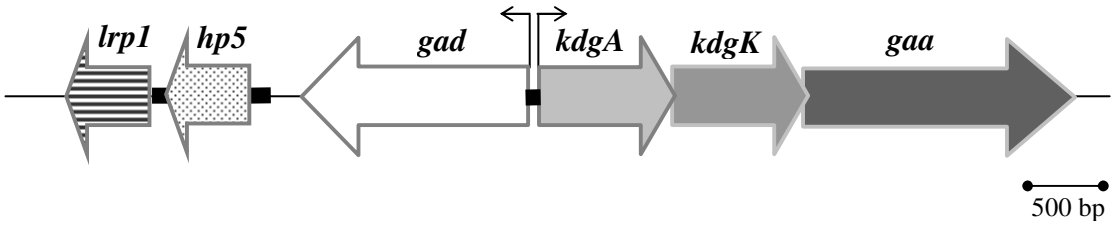
3.2.1 Genome organisation

The Entner-Doudoroff (ED) gene cluster of *T. tenax* consists of the *kdgA-kdgK-gaa* operon, encoding 2-keto-3-deoxy-(6-phospho)-gluconate (KD(P)G) aldolase, KDG kinase, glucan-1.4-α-glucosidase (GAA) and the oppositely directed *gad* gene, coding for gluconate dehydratase (GAD; Ahmed *et al.*, 2005). Downstream (356 bp) of the *gad* gene two ORFs are located, coding for a conserved hypothetical protein (HP5, TTX_1155) and a Lrp homolog (TTX_1154; COG1522 AsnC/Lrp family of regulators) that are divided by 36 bp.

Helix-turn-helix (HTH) prediction using NPS@ (Network Protein Sequence Analysis; Combet *et al.*, 2000) suggested an approximately 71% probability that the *T. tenax* Lrp (Lrp1) contains a HTH DNA-binding motif. The sequence at this position (18-39) is KTLQELAEAVNRPKTTIASRIK (see appendix for whole *lrp* and *hp5* nucleotide- and amino acid sequence information; Fig. A1). Furthermore, TTX_1154 shows high similarity (25 % aa identity) to the characterised LrpA of *Pyrococcus furiosus* (PF1601), which negatively regulates its own transcription (Brinkmann *et al.*, 2000; Dahlke and Thomm, 2002). For the conserved hypothetical protein (HP5, 74% aa identity to conserved hypothetical protein of *P. aerophilum* (PAE3481)) no HTH DNA-binding motif was detected by NPS@.

Due to the close clustering of the ORFs belonging to the ED gene cluster (see fig. 3.8) with the ORFs TTX_1154 and TTX_1155, a functional relation was supposed.

A



lrp1 leucine-responsive regulatory protein (Lrp1; TTX_1154; 504 bp)
hp5 hypothetical protein (HP5; TTX_1155; 459 bp)
gad gluconate dehydratase (GAD; TTX_1156; 1,176 bp)
kdgA KD(P)G aldolase (KD(P)GA; TTX_1156; 861 bp)
kdgK KDG kinase (KDGK; TTX_1157; 933 bp)
gaa glucoamylase (GAA; TTX_1158; 1,821 bp)
■ Putative promoter region *hp5* and *lrp*
→ Transcription start site, determined by primer extension analyses (Ahmed *et al.*, 2005)

B

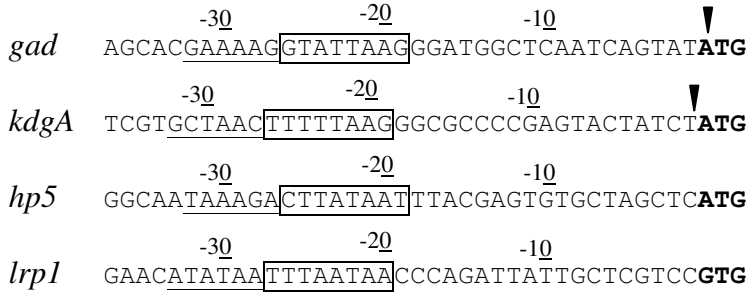


Fig. 3.8 **Clustering of the ED gene with putative transcriptional regulators. A)** Genes and their orientation are shown as arrows. The key for genes is given below. Promoter regions are indicated (black bars). **B)** The promoter region of the ED genes and the putative promoters of the *lrp1* and *hp5* genes are given. The transcript starts of the *gad* and the *kdgA* were determined by primer extension analyses (Ahmed *et al.*, 2005). The putative promoter elements BRE site (underlined) and TATA box (boxed) as well as the start codon (bold), are marked.

In order to analyse a regulatory function of TTX_1154, encoding the Lrp-like regulator and TTX_1155 coding for the hypothetical protein (HP5), in the transcription of the ED genes, the *lrp* and *hp*5 genes were cloned and heterologously expressed in *E. coli*. The recombinant proteins were analysed for their DNA-binding capacity performing electrophoretic mobility shift assays (EMSAs, see 3.2.3). PCR-amplified DNA probes spanning the predicted promoter regions of *lrp*, *hp5* as well as of the ED gene cluster (see fig. 3.8) were used as target sequences.

3.2.2 Cloning and heterologous expression of *T. tenax* Lrp1 and HP5 in *E.coli*

The *hp5* (459 bp) and the *lrp* (504 bp) gene were amplified via PCR mutagenesis employing *Pfu* DNA-polymerase using 100 ng genomic *T. tenax* DNA as template and the primer sets *hp5-Nco*I-f / *hp5-BamH*I-rev and *lrp1-Nco*I–fII / *lrp1-BamH*I-revII, respectively (see tab. 2.2) (PCR settings: 2 min 94°C; 30 cycles of 1min 94°C / 1min 63°C / 1 min 72°C; 10 min 72°C).
For recombinant expression using the pET system, the amplified *hp5* and *lrp* genes were cloned into pET15b and sequence was checked by automated dideoxy sequencing (see 2.5.10.1). The recombinant vector molecules pET15b-*lrp1* and pET15b-*hp5* were used to transform to *E. coli* Rosetta(DE3) and expressed (see 2.3 and 2.8.1).
The recombinant proteins were enriched by heat precipitation at 70°C and 80°C for 30 min (see 2.8.3.4).

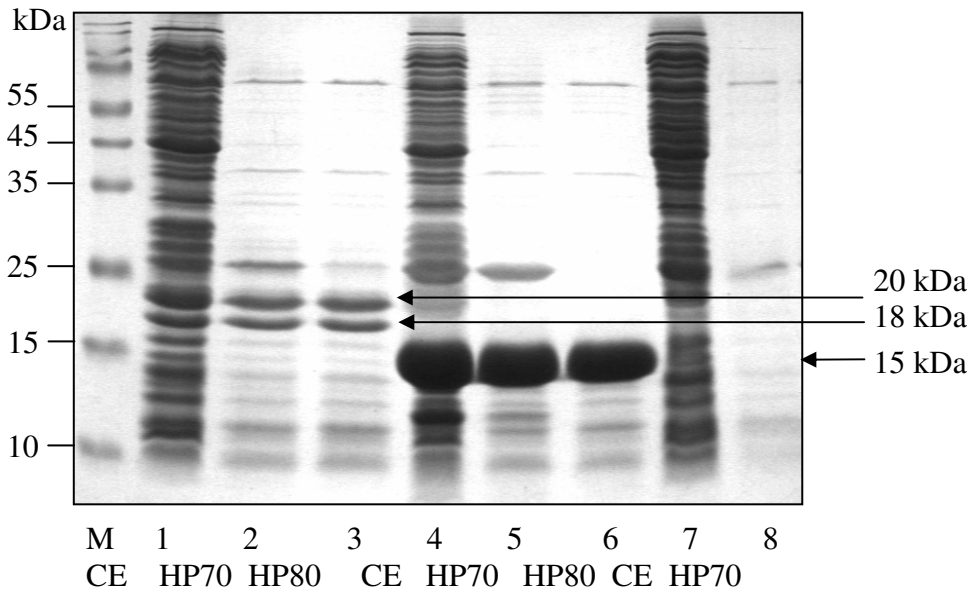


Fig. 3.9 **SDS gel electropherogram of the recombinant putative transcription regulators Lrp1 and HP5 in *E. coli*.** M: protein standard; lanes 1-3: Rosetta(DE3) pET15b-*lrp*, CE: crude extract, HP70, HP80: soluble fraction after heat precipitation at 70°C and 80°C, respectively. Lanes 4-6: Rosetta(DE3) pET15b-*hp5*, CE: crude extract, HP70, HP80: soluble fraction after heat precipitation at 70°C and 80°C, respectively. Lanes 7 and 8: Rosetta(DE3) pET15b cells (control), CE: crude extract, HP70: soluble fraction after heat precipitation at 70°C (12.5 % PAA gel).

Two dominant protein species occurred in the Lrp1 preparations (see fig. 3.9). The molecular mass of both species (18 and 20 kDa, respectively) approximately corresponds to the theoretical mass of Lrp1 (18.4 kDa).

For the recombinant HP5 a molecular mass of about 15 kDa was obtained from the SDS PAGE (see fig. 3.11) corresponding well to a theoretical mass of the protein (16.2 kDa). Both were enriched by heat precipitation in sufficient amount (Lrp1: 1.5 mg/1g cells, HP5: 3.2 mg/1g cells).

For further analysis of the different Lrp1 protein species, the *lrp* gene was cloned with a N-terminal histidine-tag (see tab. 2.2) into the vector pET24a and purified from heat precipitated crude extract HP80°C) via Ni-chelating affinity chromatography as described previously (see 2.8.3.5). Surprisingly, three bands occurred in the elution fraction (see fig 3.10).

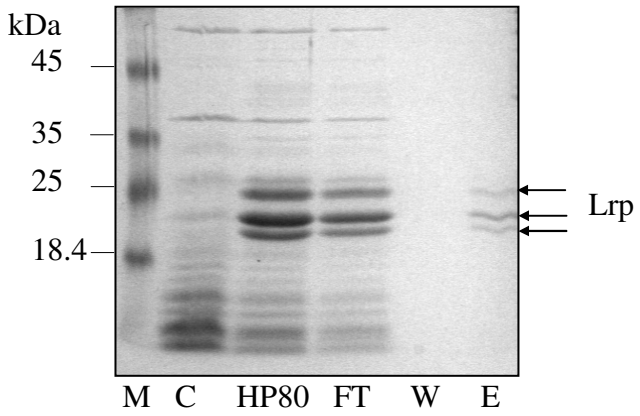


Fig. 3.10 **Purification of the recombinant Lrp1 via His tag-specific affinity chromatography.** SDS PAGE (15% PAA gel); M: protein standard; C, control: *E. coli* Rosetta(DE3) pET24a, soluble fraction after heat precipitation at 80°C; *E. coli* Rosetta(DE3) pET24a-*lrp1,* HP80: soluble fraction after heat HP at 80°C, FT: flow-through after application on Ni-NTA column; W: washing fraction; E: elution fraction (elution buffer containing 250 mM imidazole).

As a possible explanation for the appearance of different Lrp1 species a posttranslational modification of the recombinant protein has been considered, which influences electrophoretic mobility in the SDS-PAGE. To test, whether phosphorylation of Lrp1 causes different mobility in the SDS-PAGE, the recombinant protein was incubated with calf intestinal alkaline phosphatase (CIAP; 2 U) at 37°C as described previously (see 2.8.3.1). However, no mobility change of one of the protein species was observed.

3.2.3 DNA binding studies using Electrophoretic Mobility Shift Assays (EMSAs)

Generation of the DNA probes was performed via PCR amplification and subsequent 3'-end-digoxigenin labelling employing terminal transferase (50 U) as described above (see 2.5.11.1). Primer sets, that were used for the amplification of the promoter spanning regions of the ED gene cluster, *hp5* and the *lrp* gene (200 bp up- and downstream of the putative promoter), are shown in table 2.4. The PCR products (about 400 bp) potentially contained the putative DNA-binding site.

50 ng of the DIG-labelled probes and 3 µg of the recombinant protein were incubated for 15 min at either RT or 37°C in TEK buffer. The DNA-protein complexes were separated from unbound DNA by native PAGE followed by the transfer to a nylon-membrane (see 2.5.11.2) and finally immunological detection was carried out (see 2.5.12 and fig. 3.14).

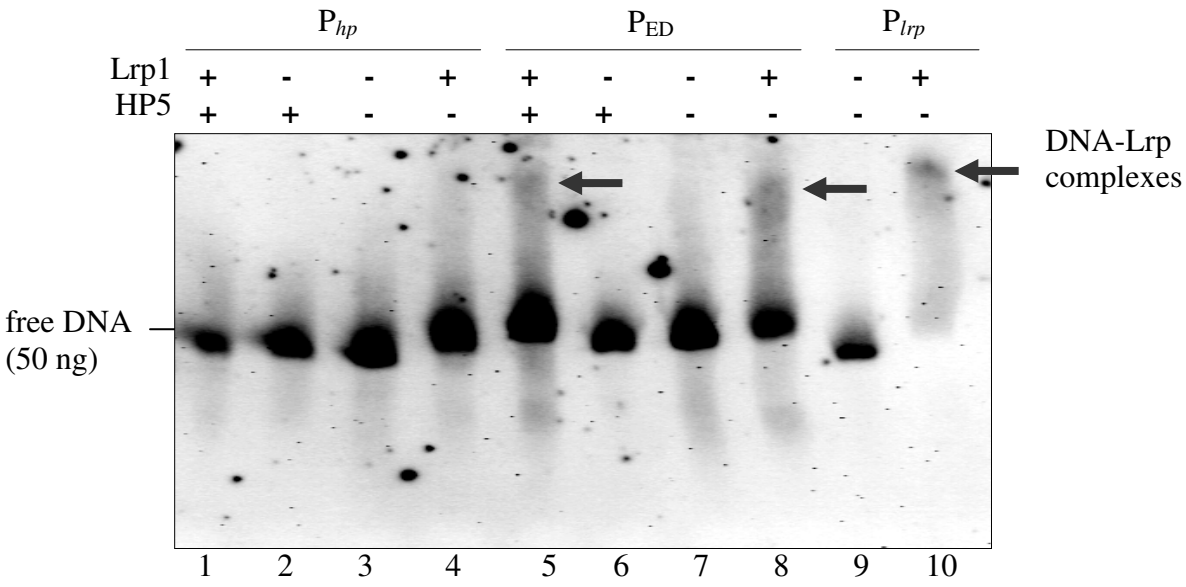


Fig. 3.11 **Electrophoretic mobility shift assay (EMSA) with Lrp1 and HP5 using *lrp, hp5* and the ED promoter spanning DNA regions as probes.** On top the addition of Lrp1 and HP5 (3µg) as well as of the respective probe (50 ng) is indicated. P_{hp}, P_{ED}, P_{lrp}: DIG-labelled probes (promoter spanning regions); Lane 10 shows a shift due to DNA-protein binding; lanes 5 and 8 show a faint shift. Incubation was performed at 37°C. Putative DNA-protein complexes are marked by arrows.

The obtained results indicate that Lrp1 binds to its own promoter region (lanes 9 and 10, fig. 3.11) as well as the promoter spanning region of the ED gene cluster (lanes 5 and 8, fig. 3.11). However, only a faint shift was observed, accounting for a rather instable binding of the Lrp1 to the ED promoter. HP5 does not bind to its own promoter region nor seems to have an influence on the shift of the DNA-Lrp1 complexes.

To prove specific DNA-Lrp1 interaction, competition experiments with 2 µg of salmon sperm DNA were performed. As shown in figure 3.12, no competition could be observed indicating specific Lrp1 binding to the DNA. The smearing of the signals may due to glycerol, that was added to the samples (10 %) and the native gel (2.5 %), in order to stabilise the instable P_{ED}-Lrp1 complex, but no improvement of the interaction was observed (see fig. 3.12).

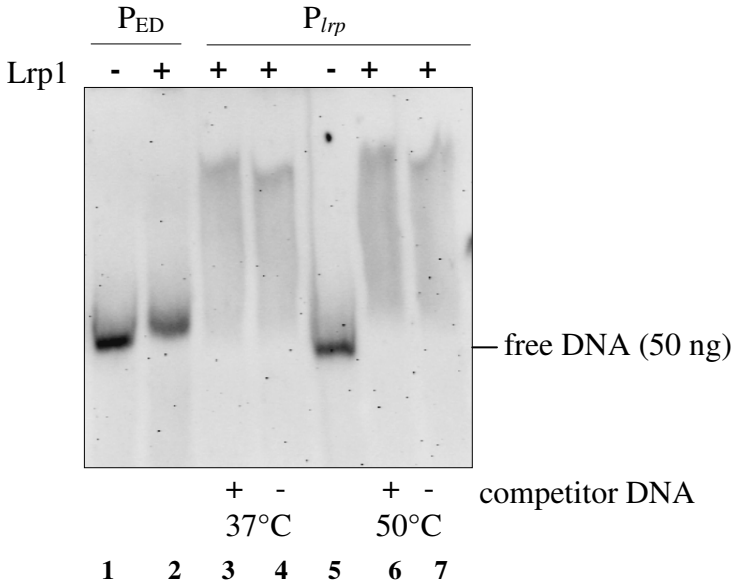


Fig. 3.12 **Electrophoretic mobility shift assay (EMSA) with Lrp1 using *lrp* and ED promoter region in presence and absence of competitor DNA.** On top the addition of Lrp1 (3µg) and of probe (50 ng) is indicated. P_{ED}, P_{lrp}: DIG-labelled probes; **Lanes 1, 2:** Lrp1 in presence of P_{ED} probe. **Lanes 3-7:** Binding of Lrp1 to its own promoter in presence and absence of competitor DNA (salmon sperm DNA) at different temperatures (37°C, 50°C).

Additionally, the influence of glucose and gluconate (substrate of the GAD) on the complex formation was checked in mobility shift assays at different temperatures (RT, 37°C, 50°C). However, no enhanced shift of the ED promoter probe in presence of the Lrp1 and the intermediates (10 mM each) was observed (data not shown).

3.3 Investigations of the trehalose metabolism of *T. tenax*

3.3.1 Genome organisation of the trehalose genes

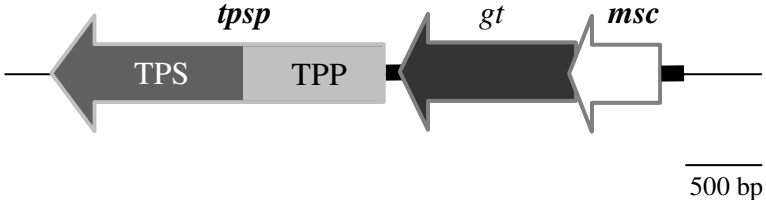
The reconstruction of the trehalose metabolism of *T. tenax* revealed the presence of the OtsA/OtsB pathway (trehalose-6-phosphate synthase (TPS/OtsA), trehalose-6-phosphate phosphatase (TPP/OtsB); Siebers *et al*., 2004), that was so far only identified in Eucarya and Bacteria (Giaever *et al.*, 1988; Kaasen *et al.*, 1994). In this pathway trehalose is formed via trehalose 6-phosphate from UDP-glucose and glucose 6-phosphate catalysed by the TPS and TPP, respectively.

In the genome of *T. tenax*, only one gene homolog was identified (*tpsp*; TTX_1304), which codes for the trehalose-6-phosphate synthase/phosphatase (TPSP), a fusion protein with a N-terminal TPS and a C-terminal TPP domain (see fig. 3.13).

Northern blot analyses revealed that the *tpsp* gene forms an operon with two upstream located ORFs (see fig. 3.13). Mono-, bi- and tricistronic RNA was detected using domain-specific *tps*- and *tpp*- as well as *gt*-specific antisense RNA probes (Zaparty, 2003).

The ORF TTX_1305 encodes a putative glycosyl transferase (*gt*) with 40% similarity (aa identity) to a putative GT of *Pyrobaculum aerophilum*. TTX_1304a encodes a putative small-conductance mechanosensitive (MS) channel. The ORF shares high similarity to conserved hypothetical proteins from *P. islandicum* and *P. aerophilum* (57% and 51% aa identity; Pisl1361 and PAE1275, respectively) as well as with putative MS channels from *Methanosarcina barkeri* (27% identity; MbarA2984) and *Picrophilus torridus* (18% identity; PTO1231). Additionally, structural prediction programs (PredictProtein, SOSUI (Hirokawa *et al.*, 1998)) forecast five transmembrane (TM) segments for the putative *T. tenax* MS channel (MscTTX; see 4.4.2).

A



tpsp trehalose-6-phosphate synthase/phosphatase (TPSP; TTX_1304; 2,196 bp)
gt putative glycosyl transferase (GT; TTX_1305; 1,164 bp)
msc putative small-conductance MS channel (MscTTX;TTX_1304a; 594 bp)
putative promoter region *msc* and *tpsp*

B

```
                -30          -20          -10
msc     GGGGCTAATCAATATATATTCGCGCGGCGGGCCCAACATG

                -30          -20          -10
gt      CCATGGTCGCCGTCGTGATCGCCCGGCTCCTCAAAAGATG

                -30          -20          -10
tpsp    GGGCACAACCGTAAAAAGGCGTTCCGTGGGAGGACAAGTG
```

Fig. 3.13 **The trehalose operon of *T. tenax*. A)** Genes and their orientation are shown as arrows. The key for genes is given below. Promoter regions are indicated (black bars). **B)** Upstream regions of *msc*, *gt* and *tpsp* gene. Putative promoter elements: BRE site (underlined), TATA box (boxed) and the start codon (bold) are marked. For complete nucleotide and corresponding amino acid sequence see appendix (Fig. A2).

Putative promoter structures, binding sites of the basal transcription factors transcription initiation factor B (TFB; BRE site: A/GNA/TAAA/T) und TATA-box binding protein (TBP; TATA-Box: NTTTTAAA) (Thomm, 1996; Soppa, 1999), were identified upstream of the *msc* and *tpsp* gene. No putative promoter region was found upstream of the *gt* gene. The *tpsp* and *gt* gene are separated by 11 bp, whereas *gt* and *msc* overlap by 4 bp.

Expression studies with the gene fragments coding for the TPS domain (Brenner, 2001) and the TPP domain (Zaparty, 2003) demonstrated, that the separated domains show indeed either TPS or TPP activity. TPP activity was also shown for a TPSP mutant (Zaparty, 2003).

In the present study the complete *tpsp* gene was cloned and heterologously expressed in *E. coli* in order to approve the bifunctional character of the *T. tenax* TPSP.

Additionally, the *gt* and *msc* gene were cloned and heterologously expressed in *E. coli* as well as in *S. solfataricus* (*msc* gene). The recombinant enzymes were functionally analysed, to confirm their predicted function.

3.3.2 Cloning and heterologous expression of the *T. tenax* TPSP in *E.coli*

The *tpsp* gene (2,196 bp) was amplified via PCR mutagenesis employing *Pfu*-DNA polymerase using 100 ng genomic *T. tenax* DNA as template and the primer sets Synf-*Nde*I-2 and RevTPSP-*EcoR*I-2 as well as tpsp 24a-his-*Nde*I-F and RevTPSP-*EcoR*I-2 (see tab. 2.2). Latter primer set was used to express the recombinant enzyme with a N-terminal histidine (6 x)-tag (PCR settings: 2 min 94°C; 30 cycles of 1 min 94°C / 1 min 58°C or 50°C, respectively / 4 min 30 sec 72°C; 10 min 72°C).

For recombinant expression using the pET system, the amplified *tpsp* gene was cloned into pET24a and nucleotide sequence was checked by automated dideoxy sequencing (see 2.5.10.1). The recombinant vector molecule pET24a-*tpsp* was used to transform competent *E. coli* Rosetta(DE3) and heterologously expressed (see 2.3 and 2.8.1).

The recombinant protein was enriched by heat precipitation at 60, 70 and 80°C for 30 min (see 2.8.3.4) as well as purified via His tag-specific affinity chromatography (see 2.8.3.5).

After cell lysis and heat precipitation three bands with a molecular mass between 80 and 92 kDa occurred in the SDS gel that were absent in the control samples (see fig. 3.14). Regarding a theoretical molecular mass of 82.04 kDa of the TPSP, all three bands could represent TPSP species.

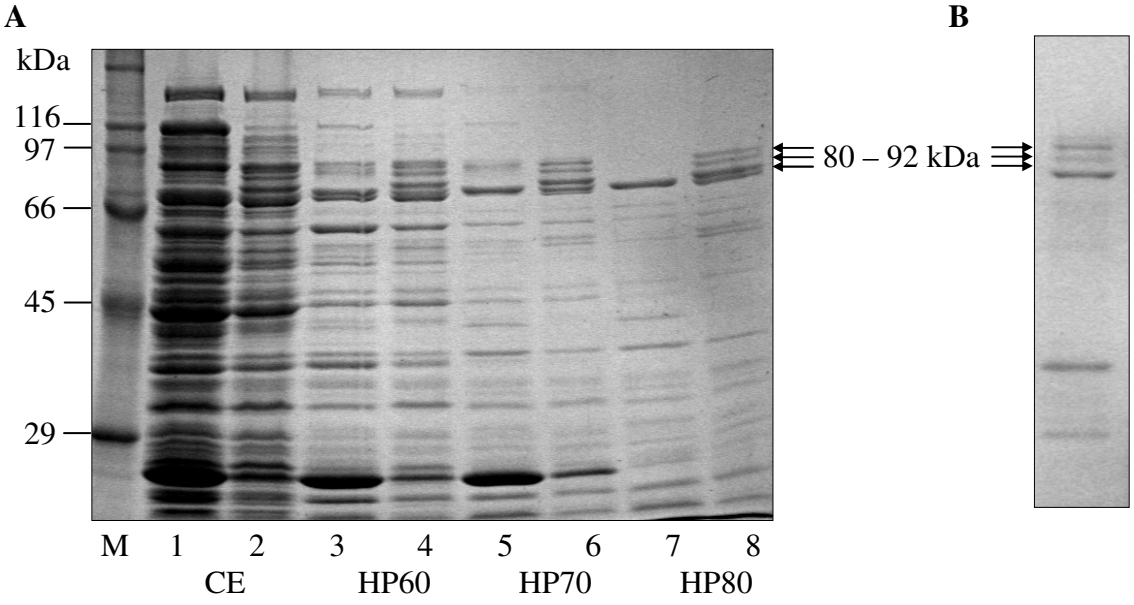


Fig.3.14 **SDS gel electropherogram of the recombinant *T. tenax* TPSP. A)** M: protein standard; lanes 1, 3, 5 and 7: Rosetta(DE3) pET24a (control); lanes 2, 4, 6 and 8: Rosetta(DE3) pET24a-*tpsp*; CE: crude extract; HP60/70/80: soluble fraction after heat precipitation at 60, 70 and 80°C, respectively. **B)** Recombinant TPSP after His tag-specific affinity chromatography. (10% polyacrylamide gel)

3.3.2.1 Western Blot analysis and determination of the N-terminal amino acid sequence

For determination of the N-terminal amino acid sequence, recombinant TPSP was enriched by heat precipitation at 90°C. Electrophoretical separation by SDS-PAGE using a 7.5 % (v/v) PAA gel revealed sufficient separation of the three TPSP isoforms. Finally, a large 7.5 % (v/v) PAA gel was used to separate the three bands and protein was transferred to a hydrophobic membrane for subsequent N-terminal sequencing (see 2.8.7.4-5 and fig. 3.15) in order to confirm the assumption that all three bands represent TPSP species.

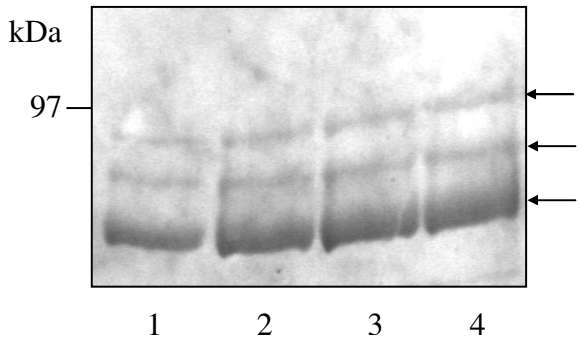


Fig.3.15 **Coomassie stained Western blot of TPSP after SDS-PAGE.** About 30 µg (lane 1), 40 µg (lane 2), 50 µg (lane 3) and 60 µg (lane 4) protein were applied on the gel. (7.5 % (v/v) PAA gel)

N-terminal sequencing, performed by Dr. R. Schmid (University of Osnabrück; see 2.8.5.5), revealed an identical N-terminal amino acid sequence over a range of the first nine amino acids (MRLIVVSNR) for all three protein species. This sequence corresponds to the deduced amino acid sequence of the TPSP (see fig. A2, appendix) as shown by N-terminal sequencing. The lower band was associated by *E. coli* DnaK (Hsp70-protein).

In order to test, whether phosphorylation of TPSP is due to the observed isoforms, the protein solution was incubated with 2 U CIAP at 37°C as described previously (see 2.8.3.1) and again checked for electrophoretic mobility. But, as already described for the recombinant Lrp1, no change of mobility was observed, suggesting that the appearance of the isoforms is not due to phosphorylation.

3.3.3 Cloning and heterologous expression of the *T. tenax* GT

The *gt* gene (1,164 bp) of *T. tenax* was amplified via PCR mutagenesis employing *Pfu*-DNA polymerase using 100 ng of a genomic fragment of *T. tenax* (clone H88; Brenner, 2001) comprising whole *gt* sequence information DNA as template and the primer sets *gt*-*Nde*I-f and g*t*-*Eco*RI-rev (see tab. 2.2). The gene was also cloned with *gt*-his-*Nde*I-f containing a histidine (6 x) tag (see tab. 2.2) and g*t*-*Eco*RI-rev, to express the recombinant protein for subsequent His tag-specific affinity chromatography (PCR settings: 2 min 94°C; 30 cycles of 1 min 94°C / 2 min 30 sec 59°C and 54°C, respectively / 1 min 30 sec 72°C; 10 min 72°C).

For recombinant expression the pET system was used and the amplified *gt* gene was cloned into pET24a. The nucleotide sequence was checked by automated dideoxy sequencing (see 2.5.10.1). The recombinant vector molecule (pET24a-*gt*) was transformed to *E. coli*

BL21(DE3), BL21(DE3) pRIL and Rosetta(DE3) and heterologously expressed (see 2.3 and 2.8.1). The recombinant protein was enriched by heat precipitation at 60, 70 and 80°C for 20 min (see 2.8.3.4) as well as via His tag-specific affinity chromatography using Ni-NTA agarose (see 2.8.3.5).

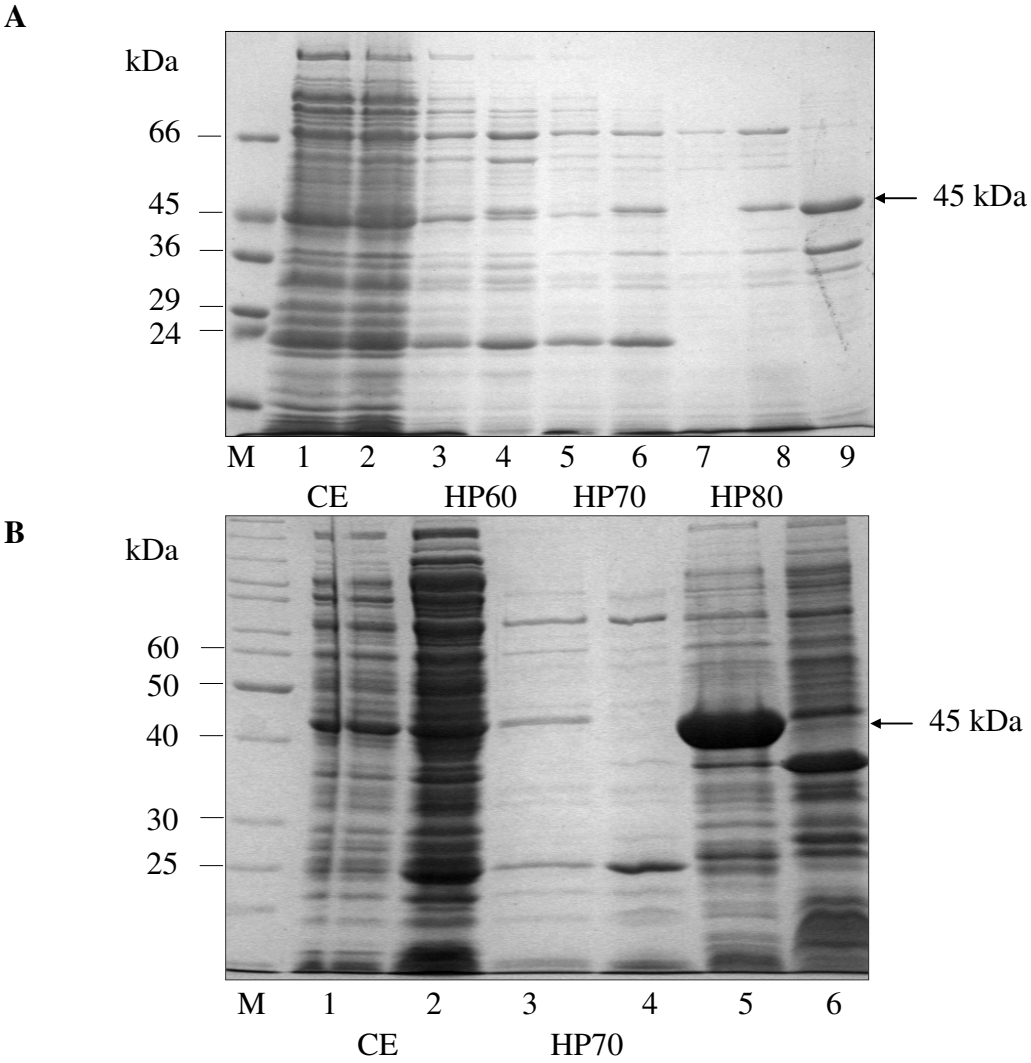


Fig.3.16 **SDS electropherogram of the recombinant *T. tenax* GT. A) Expression of *T. tenax* GT in *E. coli* BL21(DE3).** M: protein standard; lanes 1, 3, 5 and 7: BL21(DE3) pET24a (control); lanes 2, 4, 6 and 8: BL21(DE3) pET24a-*gt*; CE: crude extract; HP60/70/80: soluble fraction after heat precipitation at 60, 70 and 80°C. Lane 9: 2 µl of the BL21(DE3) pET24a-*gt* of 60,000 x *g* fraction. **B) Expression of *T. tenax* GT in *E. coli* Rosetta(DE3)**. M: protein standard; lanes 1 and 3: Rosetta(DE3) pET24a-*gt*; lanes 2 and 4: Rosetta(DE3) pET24a (control); CE: crude extract ; HP70: soluble fraction after heat precipitation at 70°C; lanes 5 and 6: 2 µl of 60,000 x *g* fraction of Rosetta(DE3) pET24a-*gt* and Rosetta(DE3) pET24a, respectively. (10% PAA gel)

After cell lysis and heat precipitation one band with a molecular mass of about 45 kDa occurred in the SDS gel, that was absent in the control samples (see fig. 3.16) and that correlated with the theoretical molecular mass of the GT (42.7 kDa).

Since, only low amount of soluble recombinant protein was expressed (0.8 mg total protein in HP70 fraction obtained from 1 g cells) the plasmid construct pET24a-*gt* was transformed to *E. coli* BL21(DE3) pRIL and Rosetta(DE3) in order to improve expression. However, no enhancement of soluble recombinant protein was achieved, but much protein was accumulated intracellularly in inclusion bodies (see fig. 3.16 B, lane 5).

3.3.3.1 I*n vitro* reconstitution of the recombinant GT from inclusion bodies

In many cases of recombinant protein expression, inclusion bodies are formed, that contain recombinant protein in mostly inactive and denatured form. However, expression in inclusion bodies has the advantages, that the recombinant protein is accumulated in high amounts and that the protein is protected from proteolytic degradation. Recombinant protein can be isolated and refolded *in vitro* by different *in vitro* reconstitution protocols. One problem constitutes the recovery of soluble, active protein in a sufficient yield.

To achieve a higher amount of soluble recombinant GT of *T. tenax*, the protein was reconstituted from the inclusion bodies by solubilisation in presence of 2 M GHCl and finally refolding through dialysis as described previously (see 2.8.3.2). After centrifugation and concentration of the soluble protein (see fig. 3.17) via spin column (< 30,000 kDa) 2 mg of recombinant GT were obtained from 5 g cells.

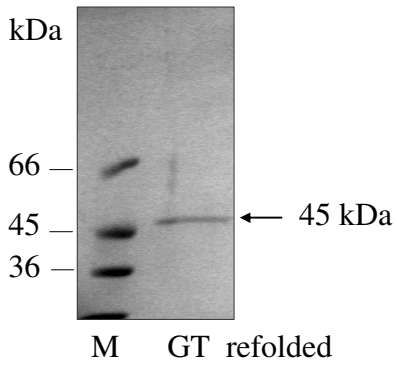


Fig.3.17 **SDS gel electropherogram of the i*n vitro* reconstituted recombinant GT of *T. tenax*.** SDS-PAGE (10% PAA gel); M: protein standard; lane 1: refolded, recombinant GT

3.3.4 Enzymatic properties of the recombinant TPSP and GT

Enzyme activities of recombinant TPSP and GT of *T. tenax* were determined by identifying the intermediates and products by thin layer chromatography (TLC).

TPSP activity was determined by a discontinuous activity assay at 70°C in the presence of the substrates UDP-glucose (UDPG, 4 mM) and glucose 6-phosphate (G6P, 8 mM) as well as MgCl2 (5 mM) and TPP activity of the TPSP was tested with trehalose 6-phosphate (Tre6P, 2 mM) in the presence of 4 mM MgCl2 as described previously (see 2.8.4).

However, in the presence of UDPG and G6P no trehalose formation was observed at 70°C (lanes 1-3, fig. 3.18), whereas Tre6P was dephosphorylated by the TPSP and thus, trehalose formation was observed (lanes 4-6, fig.3.18).

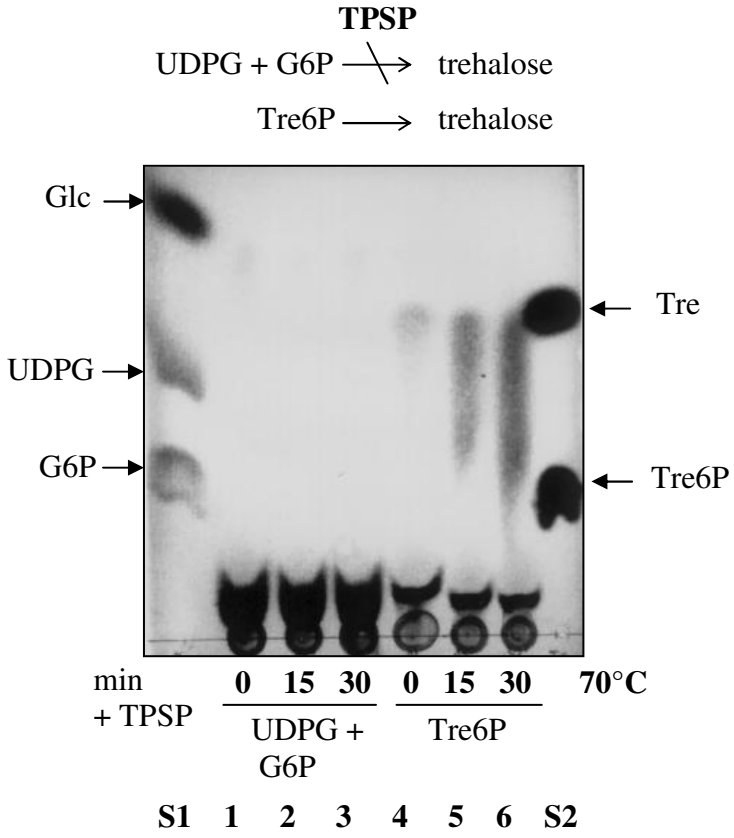


Fig.3.18 **TPSP enzyme activity identified by TLC.** 10 mM of the standards UDPG, G6P and Glc (S1) as well as Tre and Tre6P (S2) were applied; lanes 1-3: discontinuous TPSP assay, incubation at 0, 15 and 30 min at 70°C; lanes 4-6: discontinuous TPP assay, incubation at 0, 15 and 30 min at 70°C.

The same assays were performed using cell-free extract of Rosetta(DE3) pET24a (HP70°C) as control, in order to ensure that *E.coli* OtsA (TPS) and OtsB (TPP) are not active.

Furthermore, controls without substrates or lacking protein were performed. In all cases neither TPSP nor TPP activity were observed. However, using UDPG and radioactive labelled G6P in the discontinuous assay, a very low amount of trehalose was detected.
These results are confirmed by the observed activity of the single TPP domain, which showed high activity (Zaparty, 2003), whereas the single TPS domain only showed very low activity (Brenner, 2001).

The possible involvement of the recombinant putative GT in trehalose formation was investigated by the addition of the enzyme (50 µg) to the TPSP activity assay. The GT shows homology to glycosyl transferase group 1, that generally catalyse the transfer of activated monomeric sugar intermediates, e.g. nucleoside diphosphate sugar.
The incubations were performed as described previously (see 2.8.4). Surprisingly, trehalose formation was observed by the recombinant TPSP from UDPG (also ADPG) and G6P in presence of the putative GT (see fig. 3.19A).
Since trehalose 6-phosphate synthase (TPS) also represents a member of the broad glycosyl transferase family, and in order to demonstrate that the GT does not just substitute for TPS activity, trehalose formation was followed in the presence of the GT and the active TPP-domain of the truncated TPSP (see fig. 3.19 B). No trehalose formation from UDPG and G6P was observed, indicating that either activation via modification of the TPSP (e.g. phosphorylation, glycosylation) or protein-protein interaction might be involved in the activation of TPSP by GT. Trehalose formation was observed either with UDP- or ADP-glucose as substrate and $MgCl_2$ could be substituted by $MnCl_2$.
In conclusion, GT supports TPSP in catalysing the formation of trehalose from ADPG or UDPG and G6P (1), but does not substitute for TPS activity in the presence of the active, single TPP domain (2).

(1) $$\text{ADPG / UDPG + G6P} \xrightarrow{\textbf{TPSP + GT}} \text{trehalose}$$

(2) $$\text{UDPG + G6P} \xrightarrow{\textbf{TPP + GT}} \not\text{trehalose}$$

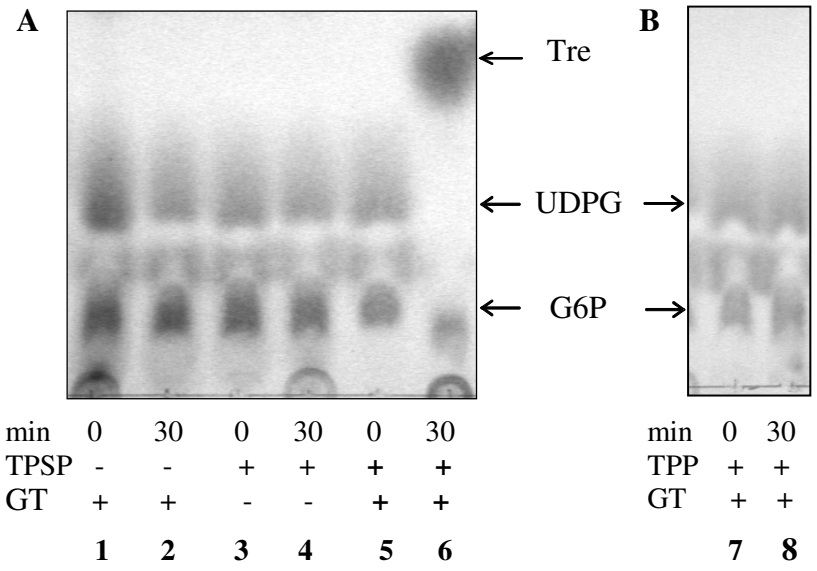


Fig. 3.19 **TPSP enzyme activity in presence of the putative GT identified by TLC. A)** Lanes 1, 2: control only containing GT, incubated 0/30 min at 70°C; lanes 3, 4: control only containing TPSP, incubated 0/30 min at 70°C; lanes 5,6: discontinuous assay containing TPSP and GT, incubated 0/30 min at 70°C; **B)** Control: no trehalose formation by the single active TPP domain and the putative GT in presence of UDPG and G6P 0/30 min at 70°C.

Furthermore it was tested, if GT enhances the activity of the single TPS domain, and if by addition of TPP trehalose is formed. Therefore the recombinant single TPS (Brenner, 2001) and the TPP domain (Zaparty, 2003) were heterologously expressed in *E. coli* and the proteins were enriched by heat precipitation (see fig. 3.20) as described for the recombinant TPSP (see 2.8.3).

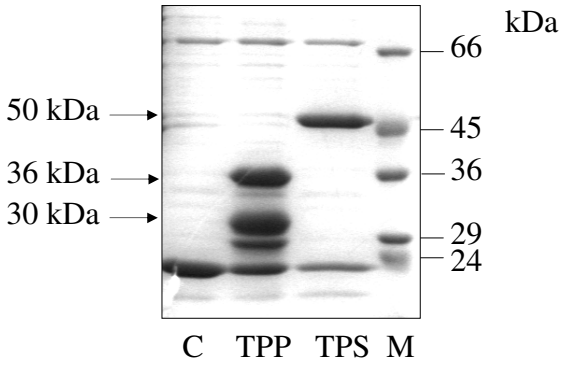


Fig. 3.20 **SDS gel electropherogram of the recombinant single TPS and TPP domain of the *T. tenax* TPSP in *E. coli*.** C: control, Rosetta(DE3) pET24a; TPP: Rosetta(DE3) pET24-*tpp*; TPS: Rosetta(DE3) pET24-*tps*; M: protein standard. Theoretical molecular mass of TPS: 53 kDa and TPP: 28 kDa. Fractions after heat precipitation at 70°C. (10% PAA gel).

However, neither trehalose 6-phosphate nor trehalose formation was observed:

$$\text{ADPG / UDPG + G6P} \xrightarrow{\textbf{TPS + GT}} \text{Tre6P}$$

$$\text{UDPG + G6P} \xrightarrow{\textbf{TPS + TPP + GT}} \text{Tre}$$

These results might depend on the very low TPS activity (Brenner, 2001) or might on the other hand point to an exclusive interaction of GT with the not truncated TPSP to form trehalose from ADPG or UDPG and G6P.

GT and TPSP were also tested for glycosyl-transferring trehalose synthase (TreT) activity, which was recently described in *Thermococcus litoralis* (Qu *et al.*, 2004). TreT catalyses the reversible synthesis of trehalose from UDPG or ADPG and glucose. Neither for GT nor TPSP the respective activity was observed.

To check for a possible modification of the TPSP by the putative GT, both recombinant proteins were incubated for 30 min at 70°C in presence of 10 mM ATP and 4 mM $MgCl_2$ in order to show possible phosphorylation of the TPSP. After incubation the samples were applied to a SDS gel (see fig. 3.21). However, no change of mobility of one of the three TPSP protein bands was observed.

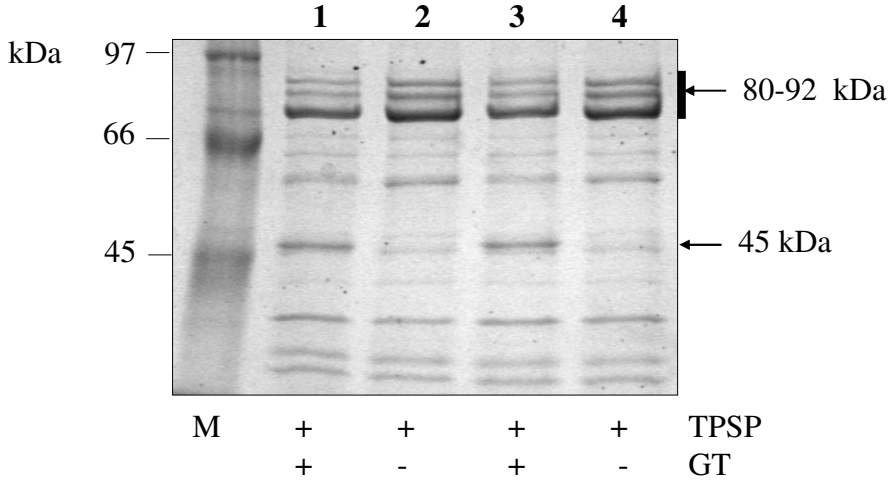


Fig.3.21 **SDS gel electropherogram of the TPSP-GT incubation assay.** M: protein standard; lane 1 and 3: 10 µg total protein of TPSP and GT, dialysed; lane 2 and 4: 20 µg TPSP, not dialysed. Fractions after heat precipitation at 70°C. (10% PAA gel)

3.3.5 Enzymatic measurements in crude extracts of *T. tenax*

Crude extracts of *T. tenax* grown autotrophically (on H_2/CO_2) and heterotrophically (on glucose) were prepared from cells harvested in exponential growth phase (6 x 10^7 cells/ml) as described previously (see 2.3).

The enzymatic assays were performed at 86°C for 60 min using dialysed and non-dialysed auto- and heterotrophic cell-free extracts (50 µg total protein; see 2.8.5).

TPSP and GT activity was shown by identification of trehalose by thin layer chromatography (see 2.8.4).

Trehalose formation from either UDPG and G6P as well as from Tre6P and thus, TPSP and GT activity was observed in both, heterotrophic and autotrophic cell-free extracts of *T. tenax* (see fig. 3.22). No difference was observed between dialysed and non-dialysed cell-free extracts. Negative controls without substrate and crude extract were performed, revealing no trehalose formation.

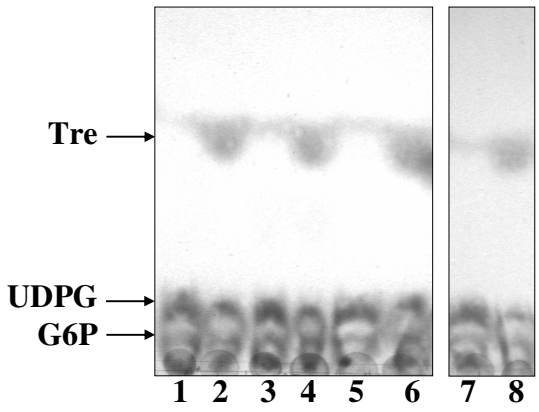


Fig. 3.22 **TPSP and GT activity in cell-free extracts of *T. tenax* identified by TLC.** Lanes 1-4: Discontinuous TPSP-GT assay performed with autotrophic crude extracts (CE, 50 µg protein): 1: 0 min and 2: 60 min at 86°C performed with dialysed CE; 3: 0 min and 4: 60 min at 86°C with non-dialysed CE; Lanes 4-8: Discontinuous TPSP-GT assay performed with of heterotrophic CE (50 µg): 5: 0 min and 6: 60 min at 86°C performed with dialysed CE; 7: 0 min and 8: 60 min at 86°C with non-dialysed CE.

3.3.6 Cloning and heterologous expression of the putative *T. tenax* MS channel

3.3.6.1 Heterologous expression in *E.coli*

The *msc* gene (594 bp) of *T. tenax* coding for the small conductance mechanosensitive (MS) channel of *T. tenax* was amplified via PCR mutagenesis employing *Pfu*-DNA polymerase

using 100 ng of a genomic fragment of *T. tenax* (clone H88; Brenner 2001) comprising whole *msc* sequence information as template and using the primer set *hp-Nde*I-f and *hp-Eco*RI-rev (see Tab. 2.2; PCR settings: 2 min 94°C; 30 cycles of 1 min 95°C / 1 min 30 sec 60°C / 1 min 72°C; 10 min 72°C).

For recombinant expression in *E. coli* using the pET system, the amplified *msc* gene was cloned into pET24a and sequence was checked by automated dideoxy sequencing (see 2.5.10.1). The vector construct pET24a-*msc* was transformed to *E. coli* Rosetta(DE3). However, the heterologous expression of recombinant putative Msc revealed problems: After induction by the addition of IPTG (1 mM, at OD_{578} ~0.6) growth of the host was strongly inhibited (see fig. 3.23). Also lowering of growth temperature and reducing IPTG to 0.5 mM to slow down expression of Msc, did not result in better growth. Obviously, the *msc* gene product exhibits toxic effects for the host.

Therefore, the *msc* gene was amplified via PCR mutagenesis using *hp-Nde*I-f and *msc*-his-N-*Xho*I-rev as well as *hp-Nde*I-f and *msc*-his-C-*Xho*I-rev (see tab. 2.2) in order to express the recombinant protein with a N- or C- terminal histidine(his)-tag, respectively. The two amplified *msc* PCR products were cloned into pET24a for heterologous expression with a C-terminal and into pET15b for expression with N-terminal his-tag, respectively. Furthermore, a vector (pTRCH6), which is used for over-expression of MsCS of *E. coli* (kindly provided by Prof. I. Booth, University of Aberdeen (Scotland, UK)), was used for the expression of the *T. tenax* Msc.

All three constructs (pET24a-*msc*-C-his, pET15b-*msc*-N-his, pTRCH6-*msc*-C-his) were transformed to *E. coli* BL21(DE3) containing pLys. The plasmid encodes T7 lysozyme, a natural inhibitor of the T7 polymerase. Lysozyme is constitutively expressed and thus, minimises low-level expression of the toxic Msc before IPTG induction.

Although cell growth of *E. coli* BL21(DE3) pLys (containing pTRCH6-*msc*-C-his, pET24a-*msc*-C-his or pET15b-*msc*-N-his) was not as dramatically influenced as in case of Rosetta(DE3) (see fig. 3.23), no recombinant Msc protein could be enriched.

Attempts to partially rescue the *E. coli* cells by increasing osmolarity of the medium (Kloda and Martinac, 2001c), were only less successful. In presence of 300 mM KCl, 300 mM NaCl or 600 mM sorbitol, growth was partially rescued (see fig. 3.23), however, no recombinant protein was found either in the membrane or in the soluble fraction.

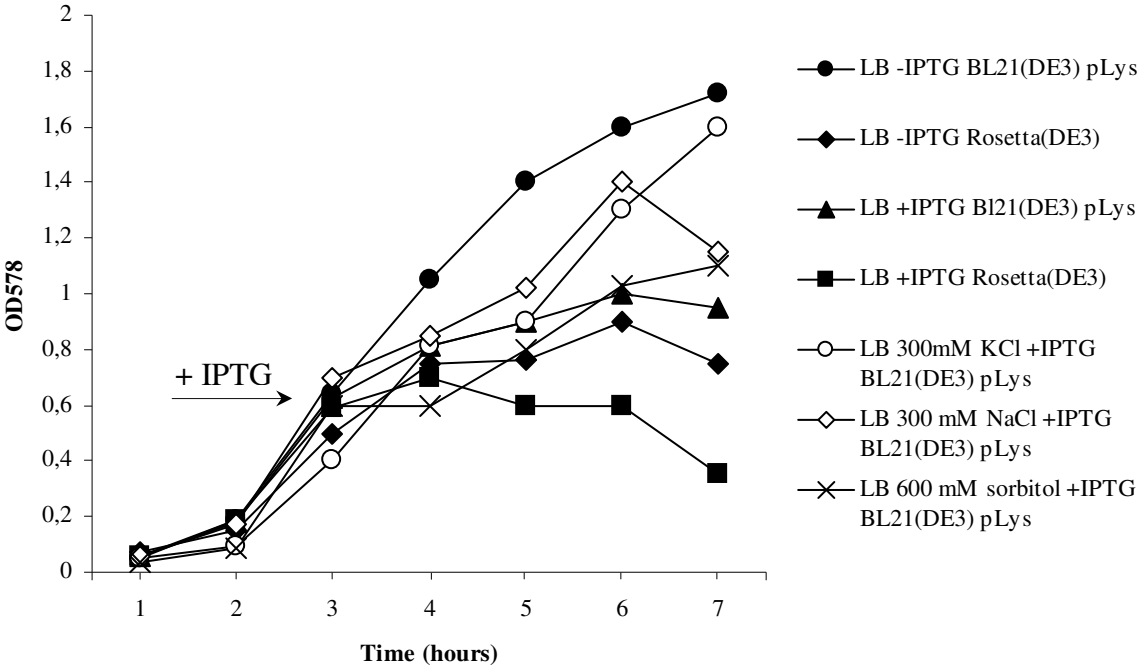


Fig: 3.23 **Effect of expression of the putative Msc of *T. tenax* on growing *E. coli cultures.*** Growth of *E. coli* Rosetta(DE3) (■) and BL21(DE3) pLys (▲) growth was inhibited after induction of MscTTX expression by adding IPTG (1 mM) at OD_{578} ~0.6 (marked with an arrow). Growth was partially rescued in media of high osmolarity containing either 300 mM KCl (○), 300 mM NaCl (◊), or 600 mM sorbitol (×).growth of non-induced BL21(DE3)pLys culture, harbouring pET24a-*msc*C-his (●), and growth of non-induced Rosetta(DE3) pET24a-*msc* (♦).

3.3.6.2 Heterologous expression of the *T. tenax* Msc in *Sulfolobus solfataricus*

For recombinant expression in *S. solfataricus*, the *msc* gene was amplified via PCR mutagenesis employing *Pfu*-DNA polymerase using 100 ng of genomic *T. tenax* DNA as template and the primer set *msc-Bsp*HI-f and *msc-BamH*I-rev (see Tab. 2.2; PCR settings: 2 min 94°C; 30 cycles of 1 min 94°C / 1 min 30 sec 60°C / 1 min 72°C; 10 min 72°C).

The construct was cloned into the vector pMZ1 (kindly provided by Dr. S.V. Albers, University of Groningen (NL)) in order to express the putative Msc with a C-terminal tandem-tag (streptavidin**-**histidine(6x)-tag) for subsequent purification and detection. Sequence was checked by automated dideoxy sequencing (see 2.5.10.1).

Following steps were performed by Dr. S.V. Albers (University of Groningen (NL)). The preconstruct pMZ1-*msc* was cloned into the shuttle vector pMJ03 (modified; Jonuscheit *et al*., 2003; Albers *et al.*, 2006) and the resulting expression plasmid pSVA80 was used for transformation of electroporated *S. solfataricus* PH1-16 (Δ*pyrEF*) cells, which subsequently

were grown in 5 l selective medium (lacking uracil). Induction was performed by the addition of 0.4% arabinose (at OD_{578} 0.3), expression was carried out for 12 h and the cells were harvested by centrifugation.

After solubilisation of the membrane, Msc containing the C-terminal strep-his-tag was purified via His tag-specific affinity column, afterwards blotted and stained with streptactin (see fig.3.24).

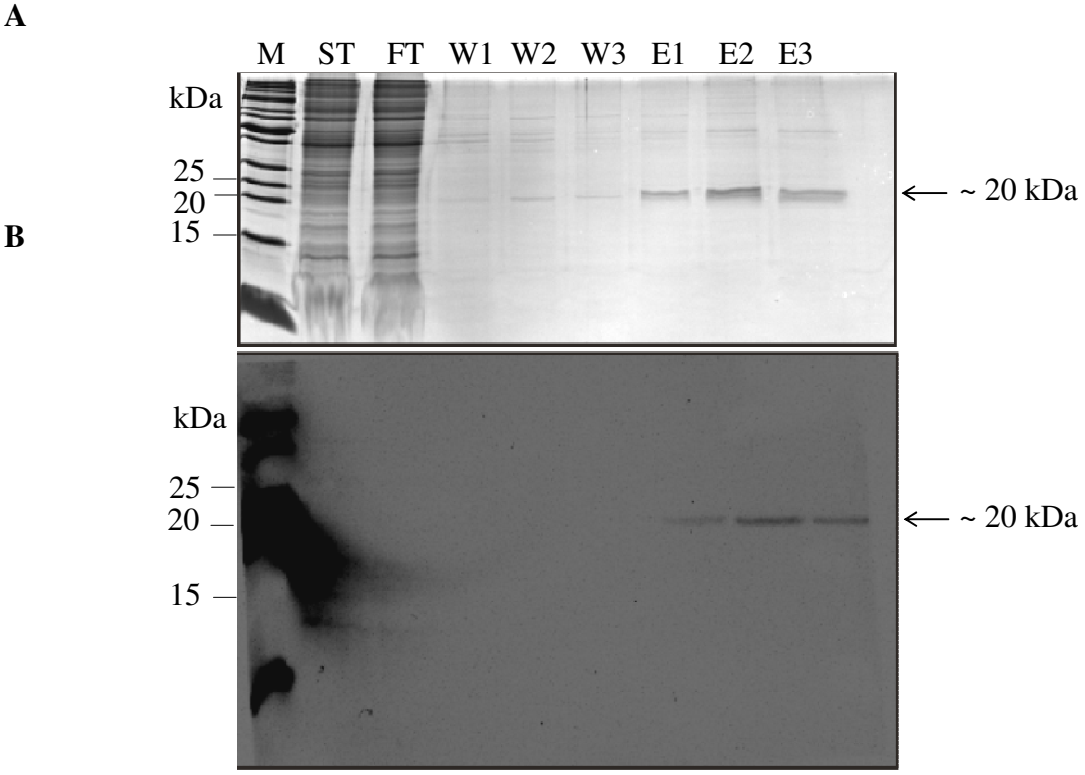


Fig: 3.24 **SDS gel electropherogram and stained Western blot showing purification of the heterologously expressed putative *T. tenax* Msc in *S. solfataricus*. A)** Coomassie stained gel of His tag-specific affinity chromatography fractions (His-Select column). M: protein standard; ST: starting fraction: solubilised membrane fraction; FT: flow through, W1-3: Washing fractions, E1-3: Elution fractions **B)** Detection of the blotted, recombinant *T. tenax* Msc using streptactin, revealing a protein of about 20 kDa. This work was performed by Dr. S.V. Albers, University of Groningen (NL).

4 DISCUSSION

4.1 Reliability of the microarray data

The microarray experiments were performed with 14 independent *T. tenax* cultures, seven grown heterotrophically on glucose and seven grown autotrophically on carbon dioxide and hydrogen, respectively. The result given for each open reading frame (ORF) represents an average of five hybridisation experiments. Additionally, a control experiment comparing two independent cultures both grown autotrophically on carbon dioxide was performed that revealed no changes in gene expression more than twofold for any of the ORFs (see 3.1.5) and therefore confirmed the reliability of the derived microarray data.

Performing transcriptional profiling using the focussed *T. tenax* CCM microarray, internal standardisation was chosen as method for normalisation of the microarray data. The *rpoS* gene of *E. coli*, coding for the stationary phase sigma factor was used as an internal standard, because sigma factors are not involved in archaeal transcription. Normalisation by internal standardisation avoids methodical differences, e.g. due to differential dye incorporation, as described previously (Zaigler *et al.*, 2003).

Finally, the performed Northern blot analyses of six selected ORFs verified the obtained microarray results (see 3.1.6).

4.2 Adaptations of the *T. tenax* CCM to different carbon sources

Variability within central metabolic pathways of Archaea is documented very well by their glycolytic pathways. Archaeal sugar metabolism shows several unique features characterised by a great variety of novel archaeal enzymes (Ronimus and Morgan, 2003; Verhees *et al.*, 2003; Sakuraba *et al.*, 2004; Siebers *et al.,* 2004; Siebers and Schönheit, 2005; van der Oost and Siebers, 2007). Recent studies of sugar metabolism in hyperthermophiles revealed the presence of unusual metabolic processes, e.g. a modified version of the Embden-Meyerhof-Parnas (EMP) pathway or the branched Entner-Doudoroff (ED) pathway in *T. tenax* (Siebers and Hensel, 1993; Selig *et al.*, 1997; Siebers *et al.,* 1997; Siebers *et al.*, 2004; Ahmed *et al.*, 2005). However, although *T. tenax* utilises both pathways for glucose catabolism simultaneously as shown by *in vivo* NMR studies, it was found that the EMP pathway

represents the main route for glucose degradation in *T. tenax* (Siebers *et al.*, 1997).

As shown for the EMP pathway, allosteric regulation at protein level, which seems to play an important role in the classical EMP pathway found in Bacteria and Eucarya seems to be reduced in Archaea. It has been previously suggested that regulation on gene level might plays an important role in the control of central carbohydrate metabolism (CCM; Verhees *et al.*, 2003, Schut *et al.*, 2003, Zaigler *et al.*, 2003).

Because of its metabolic versatility, *T. tenax* represents a suitable model organism to study the regulation of the glycolytic/gluconeogenic switch of CCM. Further on, the genome sequence information as well as biochemical information on several enzymes of the CCM are available, which were used previously for the reconstruction of the respective CCM pathways (Siebers *et al.*, 2004).

Compared to the knowledge about archaeal CCM complexity and its modifications, information on the regulation of CCM, is rather scarce. The present focussed transcriptional profiling of the *T. tenax* CCM in response to changes of the carbon source (glucose vs CO_2) contributes to further elucidation of CCM regulation not only in *T. tenax* but also in Archaea, in general.

4.2.1 The reversible EMP pathway

In contrast to the classical version of the EMP pathway known from Bacteria and Eucarya the classical regulation sites at the beginning of the pathway, represented by the enzyme couples hexokinase / glucose-6-phophate phosphatase, phosphofructokinase / fructose-1,6-bisphosphatase are absent in *T. tenax*. The EMP variant of *T. tenax* is characterised by an ATP-dependent hexokinase (ATP-HK; Dörr *et al.*, 2003) and a reversible pyrophosphate-dependent phosphofructokinase (PP_i-PFK; Siebers *et al.*, 1998), both of which show no notable regulatory properties. Furthermore, three different GAP-converting enzymes were identified: The highly allosteric, non-phosphorylating GAP dehydrogenase (GAPN; Hensel *et al.*, 1987, Brunner *et al.*, 2001), the classical phosphorylating GAPDH (Brunner *et al.*, 1998, Brunner *et al.*, 2001) and a non-phosphorylating ferredoxin(Fd)-dependent GAP oxidoreductase (GAPOR). Finally, also phosphoenolpyruvate (PEP)/pyruvate conversion in *T. tenax* is accomplished by three different enzymes: A catabolic pyruvate kinase (PK) with only a very low regulatory potential (Schramm *et al.*, 2001), the reversible pyruvate

phosphate dikinase (PPDK), which according to its biochemical properties represents a catabolic enzyme (Tjaden *et al.*, 2006), and the anabolic phosphoenolpyruvate synthetase (PEPS; Tjaden *et al.*, 2006). Therefore, the classical control points of this pathway found in Bacteria and Eucarya are absent in *T. tenax* and Archaea as confirmed by other studies in general.

Interestingly, phosphorylation of glucose catalysed by an ATP-dependent hexokinase with reduced allosteric potential is not regulated on transcript level in response to the offered carbon source. However, the *hxk*-gene was shown to be co-transcribed with a small open reading frame (*orfX*) of unknown function and whereas the monocistronic *hxk* transcript was observed under heterotrophic growth conditions only the bicistronic transcript was detected under autotrophic growth conditions, thus suggesting a regulation by RNA processing (Dörr *et al.* 2003).

The increased transcript levels of the *fba-pfp* operon coding for the PP_i-PFK and the FBPA under glucose growth corresponds nicely to the determined increase in enzyme activity in heterotrophically grown cells, respectively (Siebers, 1995). This finding is surprising since both enzymes catalyse per se reversible reactions. The reversible PP_i-PFK substitutes for the antagonistic, unidirectional enzyme couple ATP/ADP-PFK and FBPase type V found in all other Archaea using a reversible EMP modification.

The unusual archaeal type Class I FBPA was shown to catalyse the reversible cleavage of fructose 1,6-bisphosphate and a slight (2.2-fold) activation by saturating concentrations of citrate (10 mM citrate) was observed (Siebers unpublished data, Siebers *et al.* 2001).

The significant induction of expression of the *fba-pfp* operon in correlation with elevated enzyme activities strongly suggests higher catabolic fluxes under heterotrophic growth conditions.

At the level of GAP conversion, the GAPOR catalyses like GAPN the irreversible, non-phosphorylating oxidation of GAP yielding 3-phosphoglycerate, however, using ferredoxin instead of pyridine nucleotides as co-substrate. Whereas, the GAPN of *T. tenax* is well characterised and was shown to exhibit allosteric properties (Brunner *et al.*, 1998, Brunner *et al.*, 2001), no biochemical information is available for the GAPOR of *T. tenax*. However, the enzyme of *P. furiosus*, which shares 37 % identity to the *T. tenax* enzyme was characterised in great detail (Mukund and Adams 1995, Van der Oost *et al.* 1998) and no allosteric properties but a significant up-regulation of transcript amounts in response to heterotrophic growth conditions on cellobiose (Van der Oost *et al.*, 1998) was reported. In *T. tenax* the GAPN is regulated by the energy charge of the cell, early intermediates of the EMP pathway

as well as intermediates of glycogen metabolism and no regulation is observed on transcript level. The significant up-regulation of GAPOR transcript levels under growth on glucose therefore seems to enhance the catabolic carbon flow, through the pathway. The enzyme generates reduced ferredoxin for energy conversion and thus, probably represents a standby enzyme.

In anabolic (gluconeogenic) direction the enzyme couple GAPDH and PGK substitutes for the unidirectionally catabolic enzymes GAPN and GAPOR in *T. tenax*. Biochemical and transcriptional analyses of the classical reversible, phosphorylating GAPDH in *T. tenax* as well as *P. furiosus* revealed a true anabolic role in these organisms (Brunner *et al.*, 2001; Schaefer and Schönheit, 1993; Schut *et al.*, 2003). The significant induction of gene expression of the *gap-pgk* operon under autotrophic growth conditions confirms the proposed anabolic function of the GAPDH-PGK couple in *T. tenax*. Therefore the first and main control point in the EMP variant of *T. tenax* is executed on protein level by an allosterically regulated GAPN and additionally on transcript level by the inversely regulated genes encoding the catabolic GAPOR and the anabolic GAPDH-PGK enzyme couple.

The PEPS of *T. tenax* was characterised in detail and was shown to catalyse the unidirectional ATP-dependent conversion of pyruvate to PEP, AMP and P_i (Tjaden *et al.*, 2006), thus representing a true anabolic enzyme. In accordance with its solely anabolic function the *pps* gene is induced in CO_2-grown cells. In addition, the enzyme exhibits regulatory properties and is significantly inhibited in the presence of α-ketoglutarate, AMP and ADP suggesting reduced activity at low energy charge of the cell and under ammonia limitation. In combination with two other enzymes, the reversible pyruvate,phosphate dikinase (PPDK), which is strongly inhibited at elevated ATP concentration (1 mM ATP, 22% residual activity) and the pyruvate kinase (PK) without allosteric properties (Schramm *et al.*, 2001), the PEPS represents the second control point in the EMP variant of *T. tenax* at the level of PEP and pyruvate conversion (Tjaden *et al.*, 2006).

In summary, the induction of the genes encoding the PP_i-PFK, FBPA and GAPOR in glucose grown cells might therefore allow to enhance the carbon flux under heterotrophic growth, whereas GAPDH and PGK as well as PEPS seem to support the flux in the opposite, anabolic direction.

4.2.2 The catabolic, branched ED pathway

Comparative based genomic approaches and biochemical studies revealed the presence of the non- as well as the semi-phosphorylative ED branch (Ahmed *et al.*, 2005), the latter was by the time thought to be restricted to halophiles. The so called branched Entner-Doudoroff (ED) pathway represents the alternative route for glucose degradation in *T. tenax*.
The *T. tenax* 2-keto-3-deoxy-(6-phospho)gluconate aldolase (KD(P)GA) is a bifunctional enzyme converting KDG as well as KDPG and therefore represents a key enzyme of both ED branches. The glycerate kinase is the characteristic enzyme of the non-phosphorylative ED variant and catalyses the phosphorylation of glycerate to 2-phosphoglycerate. So far, not much is known about possible allosteric regulation of the ED enzymes of *T. tenax*.
The GK of *T. tenax* is inhibited by ADP (Kehrer *et al.*, 2007, submitted), probably representing feedback inhibition by low energy charge of the cell. The gluconate dehydratase (GAD) of *S. sulfolobus* (SSO3198) was characterised recently (Kim and Lee, 2005). It was shown that the enzyme represents a phosphoprotein and that its catalytic activity is regulated by phosphorylation – dephosphorylation.

In contrast to the EMP pathway, the ED genes exhibit no strong regulation on gene level. The finding that the gene encoding the key enzyme of the non-phosphorylative ED branch, the glycerate kinase (GK) is up-regulated under autotrophic growth conditions, is surprising, since the branched ED pathway is generally regarded as pathway for glucose degradation in Archaea. However, the encoding gene (*garK*) seems to form an operon with the ORF TTX_0789, coding for a gene homolog, which shows similarity to classical 6-phoshogluconate dehydratase (EDD, *edd;* EC 4.2.1.12) and dihydroxy-acid dehydratase (DHAD, *ilvD*; EC 4.2.1.9). The respective homolog of *S. solfataricus* was characterised recently and significant activity on dihydroxyisovalarate and gluconate was demonstrated. Therefore, a function in the biosynthesis of branched chain amino acids as well as the branched ED pathway in this organism was predicted (Kim and Lee, 2006). This hint supposes a possible functioning of the GK in amino acid metabolism, e.g. for providing the precursor 3-phosphoglycerate for the synthesis of glycine, serine and cysteine.

For the ED pathway an involvement in the hydrolytic degradation of the storage compound glycogen, beside the phosphorolytic glycogen degradation catalysed by the glycogen phosphorylase (GLGP), is assumed, because of the clustering of the ED genes (TTX_1156a

and TTX_1157, encoding KDGA and KDGK) with the ORF TTX_1158 coding for a glucoamylase (GAA), which catalyses the hydrolysis of α-1,4 glycosidic bonds and therefore releases glucose molecules. The operon organisation of these genes (*kdgA-kdgK-gaa* operon; see fig.3) suggests a functional relation of the encoded enzymes.

Another role of the ED pathway is described for some bacteria, e.g. *E. coli*, where it forms a funnel for sugar acid and polymer degradation (Peekhaus and Conway, 1998).

4.2.3 The reversible CAC

Fermentation studies and the measurement of enzyme activities in crude extract revealed that pyruvate, provided by the different glycolytic routes, is completely oxidized to carbon dioxide (CO_2) under catabolic growth conditions via the oxidative citric acid cycle (CAC; Selig and Schönheit, 1994). The *T. tenax* genome data as well as enzymatic studies in the close relatives *Thermoproteus neutrophilus* (Schäfer *et al.*, 1986; Beh *et al.*, 1993) and *Pyrobaculum islandicum* (Hügler *et al.*, 2003; Hu and Holden, 2006) suggest that CO_2 fixation in *T. tenax* proceeds via the reverse, reductive CAC. Most of the CAC enzymes catalyse easily reversible reactions and the enzyme couples citrate synthase and citrate lyase, 2-oxoglutarate dehydrogenase and 2-oxoglutarate oxidoreductase (OOR) as well as succinate dehydrogenase and fumarate reductase are supposed to work in opposite direction to control the flux through the pathway. All required CAC enzymes encoding genes, with the exception of the E2 and E3 component of the 2-oxoglutarate dehydrogenase, were found in the *T. tenax* genome, although not all genes, especially those coding for irreversible enzymes, could be assigned unequivocally (Siebers *et al.* 2004).

The transcript levels of the key enzymes of the oxidative direction, one of the two citrate synthases (CS 2; TTX_1513) and the suggested candidate for succinate dehydrogenase (TTX_0861-0864) are up-regulated in glucose-grown cells, whereas the predicted fumarate reductase (TTX_1104-1105) is up-regulated in CO_2-grown cells indicating a participation in the reductive direction of the pathway. However, no change of transcript amount was observed for the predicted citrate lyase (TTX_1435-1436-1437, β-α- γ-subunit). In addition, a significant change in transcript level was observed for one of the two identified candidates for 2-oxoglutarate oxidoreductase under autotrophic conditions, which was supposed to operate in both directions in *T. tenax*. The higher gene expression in CO_2-grown cells shows that CO_2-fixation is enhanced under autotrophic growth conditions.

Therefore it appears that the enzymes citrate synthase 2, succinate dehydrogenase and fumarate reductase as well as the reversible 2-oxoglutarate oxidoreductase function in a coordinated manner to regulate the carbon flow in the CAC of *T. tenax*. However, respective enzyme activities and their function in the reversible CAC remain to be shown.

There is no reliable evidence for an active glyoxylate shunt in *T. tenax*. The *glcB* gene encoding a putative malate synthase (MS; TTX_1316) is not expressed. Furthermore, no ORF encoding isocitrate lyase (ICL, EC 4.1.3.1), was identified in the genome of *T. tenax*.
Similar findings reveal the presence of a *glcB* and *icl* gene coding for MS and ICL in *P. islandicum*, however only MS activity was measured in crude extracts and therefore an active glyoxylate cycle is questionable (Hu and Holden, 2006). It has been supposed that MS is involved in acetate assimilation, like it was described for the bacterium *Rhodospirillum rubrum*, via citratemalate cycle (Ivanovsky *et al.*, 1997). However, *T. tenax* is suggested to be unable to grow on acetate (Zillig *et al.*, 1981).

The ORFs TTX_0328-0327-0326 with homology to the medium, small and large subunit of a carbon monoxide dehydrogenase, the key enzyme of the reductive acetyl-CoA (Wood-Ljungdahl) pathway is the only hint for an alternative CO_2 fixation pathway in *T. tenax*, however, the genes are not expressed.
Furthermore, the respective enzyme activity in cell extracts of *T. tenax* and *P. islandicum* could not be observed (Selig and Schönheit, 1994) and therefore the reductive CAC is assumed as only autotrophic CO_2 fixation pathway in *Thermoproteus* and *Pyrobaculum* (Schäfer *et al.*, 1986; Beh *et al.*, 1993; Hügler *et al.*, 2003; Siebers *et al.*, 2004; Hu and Holden, 2005).

4.2.4 Pentose phosphate metabolism

The conventional oxidative pentose phosphate pathway (OPPP), which is essential for the generation of pentoses, reducing power (NADPH) and erythrose 4-phosphate (E4P) for amino acid synthesis, is generally absent in Archaea. Beside the non-oxidative pentose phosphate pathway (NOPPP), the reversed ribulose monophosphate (RuMP) pathway was shown to be responsible to provide pentoses for anabolic purposes in most Archaea (Verhees *et al.*, 2003; Soderberg, 2005). The RuMP pathway was originally described as pathway for formaldehyde fixation in methylotrophic Bacteria, but is now recognised as a widrespread prokaryotic

pathway. The two key enzymes of the RuMP pathway, the 3-hexulose-6-phosphate isomerase (PHI) and the 3-hexulose-6-phosphate synthase (HPS), catalyse the fixation of formaldehyde with ribulose 5- phosphate (Ru5P) to form 3-hexulose-6-phosphate, which is subsequently isomerised to fructose 6-phosphate (F6P). In the reverse direction, both enzymes are able to transform F6P to Ru5P and formaldehyde, as recently shown for HPS-PHI fusion protein of *Thermococcus kodakaraensis* (Orita *et al.*, 2006) and *Pyrococcus horikoshii* (Orita *et al.*, 2005). Therefore it has been suggested that the enzymes are involved in formaldehyde fixation, as well as in the synthesis of pentoses. In the genome of *T. tenax* two ORFs, TTX_1521 (213 aa) and TTX_1049 (202 aa) have recently been identified, which code for HPS and PHI, respectively (Van der Oost and Siebers, 2007).

Erythrose 4-phosphate (E4P), precursor for the synthesis of aromatic amino acids, is gained from F6P and GAP via transketolase. In *T. tenax* two ORFs encoding the N- and the C-terminus of the transketolase (*tktA*, *tktB*; TTX_1754, TTX_1753) have been identified, which cluster with genes involved in the synthesis of aromatic amino acids.

Another metabolic link between the pentose phosphate metabolism and the CCM has been shown to be carried out by the deoxyribose-phosphate aldolase (DERA, EC 4.1.2.4), which recently was characterised from *Thermococcus kodakaraensis* (Rashid *et al.*, 2004). DERA catalyses the reversible conversion of (deoxy-)ribose-5-phosphate to GAP and acetaldehyde (AA). In the genome *T. tenax* a DERA homolog (*deoC*; TTX_0613; no. 39 fig.1) has been identified, which shows 52% identity to the DERA of *T. kodakarensis*. DERA is responsible for providing D-ribose for the synthesis of nucleotides.

For all ORFs concerning the pentose phosphate metabolism included in this study no differential or statistically significant expression is observed. Thus, indicating that the synthesis of the precursor for nucleotides and histidine (ribose 5-phosphate) as well as for the aromatic amino acids (E4P) is not influenced by the chosen growth conditions glucose and CO_2, respectively. However, respective biochemical studies on PPM in *T. tenax* are still missing.

4.2.5 Glycogen metabolism

The carbohydrate glycogen was shown to serve as carbon storage compound in *T. tenax* (König *et al.*, 1982). In the genome of *T. tenax* all homologs necessary for synthesis and degradation of glycogen are present, with the only exceptions of branching and debranching

enzyme, catalysing the formation and hydrolysis of 1,6-α bonds. Supposable, debranching activity is taken over by the glucoamylase (GAA), which forms an operon with the KD(P)GA and KDGK, underlining the close functional relation between the carbon storage compound and the catabolic ED branches.

Determination of glycogen concentration in autotrophically and glucose-grown *T. tenax* cells revealed a >10 fold higher amount of glycogen in the heterotrophic cells (Dörr, 2002). However, no change in transcription levels is observed concerning the genes involved in the synthesis and degradation of the storage compound and therefore, regulation might takes place on protein level. The GLGP of *T. tenax*, catalysing the P_i-dependent degradation of glycogen, was characterised (Dörr, 2002). It was shown that the enzyme is allosterically regulated and plays a major role in the phosphorolytic degradation of glycogen. The GLGP is inhibited by GA, F6P and particularly UDPG (intermediate; Dörr, 2002). Furthermore, there are evidences for regulation via protein phosphorylation, like it is described for eucaryal GLGP (Dörr, 2002). In the microarray analyses no tendency of differential gene expression depending on the growth conditions was observed (*P* value >0.05), supporting protein level regulation. For the ORFs TTX_1336 coding for putative dTDP-D-glucose thymidyl transferase and TTX_1335 encoding dTDP-glucose-4,6- dehydratase no signal could be detected. Maybe the respective genes are induced under other growth conditions. It has been supposed that phosphorolytic glycogen degradation functions via glycogen phosphorylase (GLGP), and that the ED pathway is responsible for the hydrolytic degradation of the storage compound, however, neither for the GLGP nor for the ED genes regulation is observed.

4.2.6 Trehalose metabolism

Trehalose has been identified in different Archaea (Martins *et al.*, 1996), however the disaccharide is widely distributed in all three domains of life and was characterised as a compatible solute in Bacteria and Eucarya (see 4.4).

Most of the genes encoding enzymes involved in the trehalose metabolism, show no statistically significant expression or most of the genes are accounted to be not expressed (see tab. 3.2). Given that trehalose is discussed as compatible solute in *T. tenax* (for detailed discussion see 4.4.1), respective genes might be induced under stress conditions, like heat, cold shock or quick changes of osmolarity. The fact that respective enzyme activities of trehalose-6-phosphate synthase/phosphatase (TPSP) and glycosyl transferase (GT) were

observed in auto- and heterotrophic crude extracts of *T. tenax* (see 3.3.5) may point to the presence of less transcript amount. The presence of transcripts generated as polycistronic RNA, in case of the putative mechanosensitive channel of *T. tenax* might be explained by posttranscriptional RNA processing of the polycistronic RNA.

4.2.7 Resume of *T. tenax* CCM regulation and key regulation sites of archaeal glycolytic pathways

Transcriptional profiling of the CCM genes of *T. tenax* reflects a highly coordinated expression of the genes involved in the reversible EMP pathway and the reversible citric acid cycle for controlling the catabolic and anabolic carbon flux. In contrast to the EMP pathway and the CAC, the genes of the catabolic branched ED pathway exhibit no strong regulation on gene level.

Transcript studies as well as available biochemical studies indicate that regulation of the EMP variant in *T. tenax* takes place on gene and on protein level. The regulation on protein level seems to be restricted to the allosteric, catabolic GAPN and the anabolic PEPS, thus, indicating that regulation on gene level plays an important role in regulation of the EMP pathway.

Under heterotrophic growth conditions the catabolic flux is enforced by the enhanced expression of the genes coding for PP_i-dependent phosphofructokinase (PFK) and fructose-bisphosphate aldolase (FBPA; *pfp-fba* operon). Additionally an up-regulation of the *gor* gene encoding Fd-dependent GAP oxidoreductase (GAPOR) is observed.

In *T. tenax* cells grown autotrophically, the genes coding for the classical, bidirectional GAP dehydrogenase, phosphoglycerate kinase (PGK; *gap-pgk* operon) as well as the phosphoenolpyruvate synthetase (PEPS) are strongly induced. Therefore, the induction of three genes (*pfp, fba, gor* in glucose grown cells; *gap, pgk, pps* in CO_2 grown cells) seems to be important for the direction of the carbon flux ("carbon switch") in *T. tenax*.

Biochemical studies revealed that the *T. tenax* EMP variant is characterised by a hexokinase as well as by the bidirectional PP_i-dependent PFK, both of which show no notable regulatory properties (Dörr *et al.*, 2003; Siebers *et al.*, 1998). Therefore, the two classical control points at the beginning of the EMP pathway, constituted by hexokinase/glucose-6-phophate phosphatase as well as the phosphofructokinase/fructose-1,6-bisphosphatase enzyme couple

are absent. Furthermore, *T. tenax* employs a pyruvate kinase (PK), which shows only a very low regulatory potential (Schramm *et al.*, 2000). However, additionally the anabolic PEPS and the reversible PPDK, both of which are regulated on protein level, catalyse the conversion of PEP/pyruvate (Tjaden *et al.*, 2006).

Combined consideration of protein and gene level regulation reveals that the main control points of the *T. tenax* EMP variant are shifted to i) the level of GAP, and ii) the level of PEP/pyruvate conversion. At the level of GAP control is performed by three GAP-converting enzymes: an anabolic GAPDH, which is non-allosteric, a catabolic, allosteric GAPN (Brunner *et al.*, 1998; 2001) and a catabolic GAPOR. Also in the regulation at the level of PEP/pyruvate conversion, three different enzymes are involved, the catabolic unidirectional PK, which is non allosteric (Schramm *et al.*, 2000), the anabolic, unidirectional PEPS, which is regulated on protein and gene level (Tjaden *et al.*, 2006), and the reversible PPDK that, according to its biochemical properties, rather represents a catabolic enzyme (Tjaden *et al.*, 2006).
In contrast to the EMP pathway, the catabolic branched ED pathway seems not to be regulated on gene level under the chosen growth conditions. Expression of the ED genes is not influenced by the given carbon sources (glucose or CO_2), with the only exception of glycerate kinase, that is induced under autotrophic growth conditions and an involvement in amino acid biosynthesis is discussed.
For the CAC enzymes a similarly coordinated gene regulation, like it has been shown for the EMP pathway, is observed. The genes coding for aconitase (ACN), isocitrate dehydrogenase (IDH) and the key enzymes of the reversible CAC, citrate synthase 2 (CS 2), reversible 2-oxoglutarate Fd-oxidoreductase (OOR) as well as succinate dehydrogenase (SDH) and fumarate reductase (FRD) are regulated on gene-level in response to heterotrophic and autotrophic growth conditions. However, no information about allosteric regulation of the involved CAC enzymes is available, yet.

So far, DNA microarray analyses comparing saccharolytic and proteolytic growth were performed for two hyperthermohilic, heterotrophic Archaea: The aerobic Crenarchaeum *Sulfolobus solfataricus* (Snijders *et al.*, 2006) and the anaerobic Euryarchaeum *Pyrococcus furiosus* (Schut *et al.*, 2003). In addition transcriptome analyses were also performed for the facultative anaerobic, moderate halophile *Haloferax volcanii* (Zaigler *et al.*, 2003).

S. solfataricus relies on the branched ED pathway (DeRosa *et al.*, 1984; Schönheit *et al.*, 1995; Ahmed *et al.*, 2005), which is promiscuous for glucose and galactose, for carbohydrate catabolism and only gluconeogenesis seems to proceed via the anabolic EMP pathway. Glucose is completely oxidised to CO_2 via an oxidative CAC with O_2 as terminal electron acceptor. In *S. solfataricus* the expression pattern of growth on yeast extract and tryptone (YT) was compared to growth on glucose. Strikingly, the results of the expression analyses of the central carbon metabolism of *S. solfataricus* on transcriptomic level revealed not a strong regulation of the respective genes and pathways (Snijders *et al.*, 2006). Only in consideration of available proteomic data, the GAPN (SSO3194) turned out to be induced under growth on glucose, furthermore the enzyme is allosterically regulated (Ahmed, 2006), whereas the PGK (SSO0527) as well as the PEPS (SSO0883) are suggested to be induced in YT grown cells and therefore, represent true anabolic enzymes (Snijders *et al.*, 2006).

The fermentative *P. furiosus* utilises a reversible modification of the EMP pathway and glucose is finally converted to acetate as main fermentation product (via ADP-forming acetyl-CoA synthetase; ACD; Musfeld *et al.*, 1999). The EMP variant of *P. furiosus* is characterised by ADP- rather than ATP-dependent kinases (glucokinase (ADP-GK), phosphofructokinase (ADP-PFK); Kengen *et al.*, 1995; Tuininga *et al.*, 1999). In glycolytic direction, GAPN and GAPOR, latter of which is regulated on gene level, catalyse the direct, non-phosphorylating oxidation of GAP to 3PG (Mukund and Adams, 1995; van der Oost *et al.*, 1998). GAPOR as well as glucose-6-phosphate isomerase (PGI), represent the key regulation points of glycoloysis in *P. furiosus* (Verhees *et al.*, 2001; Schut *et al.*, 2003). The enzyme couple GAPDH and PGK is engaged for 3PG conversion in gluconeogenesis and shown to be not active in glycolysis (Schäfer and Schönheit, 1993; van der Oost *et al.*, 1998).
Whole genome microarray analysis of *P. furiosus* was performed with cultures either grown on peptides or a carbohydrate (maltose) used as carbon sources (Schut *et al.*, 2003).
The ADP-PFK (PF1784) and GAPOR (PF0464) as well as genes encoding enzymes of the CAC (putative aconitase (PF0201), isocitrate dehydrogenase (PF0202; Steen *et al.*, 2001), citrate synthase (PF0203; Muir *et al.*, 1995)), are induced in maltose grown cells. *P. furiosus* grown on peptides revealed an induction of the fructose-1,6-bisphosphatase (FBPase, PF0613; Rashid *et al.*, 2002), PGK (PF1057) and GAPDH (PF1874; Schut *et al.*, 2003).
The conversion of fructose 6-phosphate/fructose 1,6-bisphosphate (F6P/FBP) might represent a general regulation point on gene level of EMP variants in hyperthermophiles. Gene

expression of *T. tenax* PP_i-dependent phosphofructokinase (PP_i-PFK; *fba-pfp* operon) and *P. furiosus* ADP-dependent phosphofructokinase (ADP-PFK) is strongly enhanced in heterotrophic grown cells (glucose or maltose, repectively).
Furthermore, according to *T. tenax*, GAP conversion in *P. furiosus* seems to represent the main control point of the EMP, which is also characterised by the three enzymes: anabolic GAPDH, catabolic GAPN and catabolic GAPOR. At the level of PEP/pyruvate conversion, *P. furiosus* only employs the catabolic PK and PEPS, whereas a catabolic function of the PEPS is discussed by Sakuraba *et al.* (2001) and controversely, an anabolic function is proposed for the enzyme by Hutchins *et al.*, 2001.

The facultative anaerobic, moderate halophile *Haloferax volcanii* (Zaigler *et al.*, 2003), uses a branched ED pathway, like *S. solfataricus,* and glucose is finally oxidised to CO_2 with either oxygen or nitrate as electron acceptor. The EMP pathway is active in gluconeogenic direction. Transcriptome analyses using a one fold-coverage shotgun DNA microarray were performed, to monitor gene expression changes due transition from amino acid-based to glucose-based metabolism (Zaigler *et al.*, 2003). After the shift from casamino acid medium to glucose medium four of nine ED genes were found to be induced: glucose dehydrogenase GDH, KDG kinase, KDPG aldolase, GAPDH2 and PGK.
The genes coding for GAPDH1 was repressed due to the switch of carbon source. These findings suggest that the two GAPDHs in *H. volcanii* may possess anabolic (GAPDH1) and catabolic function (GAPDH 2). Therefore, also in halophiles the control point at the level of GAP conversion seems to play a crucial role in the regulation of the glycolytic pathways. Strinkingly, the conversion of GAP and DHAP to FBP by fructose-bisphosphate aldolase is also regulated on gene level. The FBPA gene is repressed in response to glucose and therefore might indicate an important regulation site in halophiles as well.

In summary, the EMP pathway in *P. furiosus* reveals a highly regulated pathway, like it is shown for *T. tenax*, whereas the glycolytic degradation of glucose in *S. solfataricus* via the branched ED pathway, like in *T. tenax*, is not highly regulated on transcriptional level. However, for the gluconeogenic EMP pathway of *S. solfataricus* a slight up-regulation of enzymes with anabolic function (PEPS, PGK) in cells grown on yeast/tryptone medium was observed.
As expected, the genes involved in glucose degradation in *H. volcanii* were induced due to the shift from amino acid-based to glucose-based medium and repression of key genes involved

in the gluconeogenic EMP pathway was observed (GAPDH1, FBPA). Thus, the ED pathway represents an inducible, true catabolic pathway in *H. volcanii*.

The absence of allosteric sugar kinases (classical enzymes) and the presence of GAPN and GAPOR in addition to the (bidirectional) GAPDH in several hyperthermophilic Archaea, also suggested from genome information, points to a general feature of regulation of the different EMP variants in these organisms at the level of GAP (see fig. 4.1). In *P. furiosus* GAPDH, GAPN as well as GAPOR are responsible for GAP conversion, like it is shown for *T. tenax*. *S. solfataricus* employs the allosteric GAPN in catabolic and GAPDH in anabolic direction. In the halophile *H. volcanii* two GAPDHs are utilised separately, GAPDH1 for anabolic purposes and GAPDH2 in catabolism.
For *T. tenax* a second key regulatory point is located at the level of PEP/pyruvate conversion, as described previously. In contrast to *T. tenax*, only PK and PEPS, but no PPDK homolog is identified in *P. furiosus*, *S. solfataricus* or *H. volcanii.* The PEPS of *S. solfataricus* shows a slight induction in cells grown on yeast/tryptone. Also already mentioned, the function of the *P. furiosus* PEPS is not clearly shown yet.
Additionally, *T. tenax* and *P. furiosus* regulate F6P/FBP conversion and *H. volcanii* regulates gene expression of the FPBA gene, indicating that these conversions might represent general regulation sites in Archaea executed on gene level.

To sum up, the conversion of GAP seems to represent a conserved regulation point in hyperthermophilic Archaea, as shown for *P. furiosus*, *S. solfataricus* and *T. tenax* and it is even found in the halophile *H. volcanii* (see fig. 4.1).
Beside in *T. tenax*, the conversion of PEP/pyruvate might also represent, however a less conserved regulation point in the glycolytic pathways of *S. solfataricus* and *P. furiosus* (see fig. 4.1).

Hyperthermophilic Archaea

T. tenax		*S. solfataricus*	*P. furiosus*
EMP	**sp & np ED**	**sp & np ED**	**EMP**
HK PP_i-PFK FBPA	KD(P)GA KDGK GK	KD(P)GA KDGK GK	ADP-GK PGI FBPase ADP-PFK
GAPN GAPOR PGK GAPDH	GAPN GAPOR PGK GAPDH	GAPN PGK GAPDH	GAPN GAPOR PGK GAPDH
PK* PEPS PPDK	PK* PEPS PPDK	PK PEPS	PK PEPS**

Fig. 4.1 **Schematic view of the glycolytic pathways and conserved regulation sites in the hyperthermophiles *T. tenax*, *S. solfataricus* and *P. furiosus*.** sp and np ED: semi- and non-phosphorylative Entner-Doudoroff pathway, EMP: Embden-Meyerhof-Parnas pathway. Regulation on gene (shaded), on protein (underlined) and on both levels (shaded and underlined) is indicated. For *S. solfataricus* proteomic data are shown since no differential expression on transcript level has been observed (Snijders *et al.*, 2006).*Previously reported up-regulation of the PK encoding gene (Schramm *et al.*, 2000).**Regulation of the *P. furiosus* PEPS is discussed controversly. For discussion see Sakuraba *et al.*, 2001 and Hutchins *et al.*, 2001.

The finding that the hyperthermophile *T. tenax* uses modifications of the EMP as well as the two branches of the ED pathway for glucose degradation in parallel raises questions about their physiological function.

Given that the ED genes are organised in an operon comprising a homolog encoding a glucan 1,4-α-glucosidase (see fig. 3.10), the branched ED pathway is supposed to be involved in the hydrolytic degradation of the storage compound glycogen, whereas the EMP pathway is supposed to be involved in the phosphorolytic degradation of glycogen via glycogen phosphorylase.

Another aspect revealing selection of one of the different catabolic routes may rely on the energy demand of the cell. By employing the EMP pathway the net ATP gain is 1, whereas no ATP is generated by utilising the two ED branches. Therefore it has been concluded that the different glycolytic routes found in *T. tenax* do not display pathway parallelism, but rather represent the ability of the cell, to react to physiological needs (Ahmed *et al.*, 2004).

Finally, the utilisation of the different modified pathways is discussed in terms of a metabolic thermoadaptation: By engaging GAPN and GAPOR in the EMP and the semi-phosphorylative ED pathway, which catalyse the direct non-phosphorylating conversion of GAP to 3PG, the formation of the extremely thermolabile intermediate 1,3-BPG (1.5 min, 60°C) is avoided. Furthermore, by utilisation of the non-phosphorylative ED branch also the formation of other thermolabile intermediates, like GAP (14.5 min, 60°C), is prevented. Therefore, employing the non-phosphorylative ED pathway might be suitable for growth at higher temperatures.

4.3 Identification of CCM-transcriptional regulators: The leucine-responsive regulator protein (Lrp1) of *T. tenax*

Regulation at the transcriptional level appears to be an important site of regulation of archaeal metabolism, as already mentioned above.

The basal archaeal transcription machinery is closely related to the core components of the eucaryal transcription apparatus, consisting of RNA polymerase (RNAP) and transcription initiation factors. Archaea contain a typeII-like DNA dependent RNAP and homologs of several eucaryal transcription factors, including the TATA-box binding protein (TBP) and transcription initiation factor IIB (TFIIB; Bell and Jackson, 1998; Bell *et al.*, 2001).

Given that the archaeal basal transcription is similar to the eucaryal system, it could be expected that transcriptional regulators are eucaryal-like, but surprisingly, most of the so far identified transcriptional regulators are bacterial-like (Kyrpides and Ouzouni, 1999; Aravind and Koonin, 1999). Thus, archaeal transcriptional regulation represents a mosaic of bacterial and eucaryal features. How bacterial-type regulators interact and modulate transcription of an eucaryal-like transcription machinery, is therefore, a matter of particular interest.

Archaea regulate gene expression in a way similar to that of Bacteria by utilising sequence-specific transcriptional repressors and activators to either compete with RNAP for the promoter region or to stabilise its interaction with the promoter.

Several transcriptional regulators have been characterised in Archaea, e.g. the metal-dependent repressor 1 (MDR1) from *Archaeoglobus fulgidus*, a homolog of the iron-dependent bacterial repressor DxtR. MDR1 represses transcription of its own gene and of an ABC- metal transporter encoded in the same operon, in a metal dependent manner (Bell *et al.*, 1999). Furthermore, Phr, an euryarchaeal regulator of heat shock response from *P. furiosus*, specifically represses, like MDR1, transcription of its own gene, as well as that of two heat shock genes by abrogating RNAP recruitment to the promoter (Vierke *et al.*, 2002). A maltose-specific repressor (TrmB) for the trehalose/maltose transport operon from *Thermococcus litoralis* has also been characterised (Lee *et al.*, 2002).

Examples for positive gene regulation are described for *Methanothermobacter thermautotrophicus*, where Tfx is proposed to activate expression of the *fmdECB* genes coding for molybdenum formylmethanofuran dehydrogenase (Hochheimer *et al.*, 1999) and, GvpE activates the genes involved in gas vesicle formation in halophilic Archaea. The latter resembles eucaryal leucine-zipper family of eukaryotic transcriptional regulators (Krüger *et*

al., 1998; Plösser and Pfeifer, 2002), however, so far, no obvious eucaryal-type transcriptional regulators have been identified in Archaea.

A particular group of bacterial-type regulators, members of the Lrp/AsnC family (leucine-responsive regulator protein) of transcriptional regulators (COG1522), were identified in nearly all archaeal genomes (Brinkman *et al.*, 2003) and several archaeal homologs have already been characterised, e.g. LrpA from *P. furiosus* (Brinkmann *et al.*, 2000; Dahlke and Thomm, 2002), Ptr1 and Ptr2 from *Methanocaldococcus jannaschii* (Ouhammouch and Geiduschek, 2001; Ouhammouch *et al.*, 2003; Ouhammouch and Geiduschek, 2005), Ss-LrpB (Peeters *et al.*, 2004), Lrs14 (Napoli *et al.*, 1999) or LysM (Brinkmann *et al.*, 2002) from *S. solfataricus*.

Recent genome analyses revealed that members of the Lrp family of global and specific transcriptional regulators are widely distributed among prokaryotes, both Bacteria and Archaea (Brinkman *et al.*, 2003) but no homologs are found in Eucarya.

Members of the Lrp family are small DNA-binding proteins (about 15 kDa) consisting of a N-terminal domain with a helix-turn-helix (HTH) fold, usually connected by a hinge to the C-terminal effector domain. The proteins recognise specific sites in the respective promoter regions and either repress (negative) or induce (positive regulation) transcription. They specifically repress transcription of their own genes by steric hinderance of RNAP binding. It was shown for the LrpA from *P. furiosus*, that the protein inhibits transcription of its own gene by abrogation of RNAP recruitment (Dahlke and Thomm, 2002).

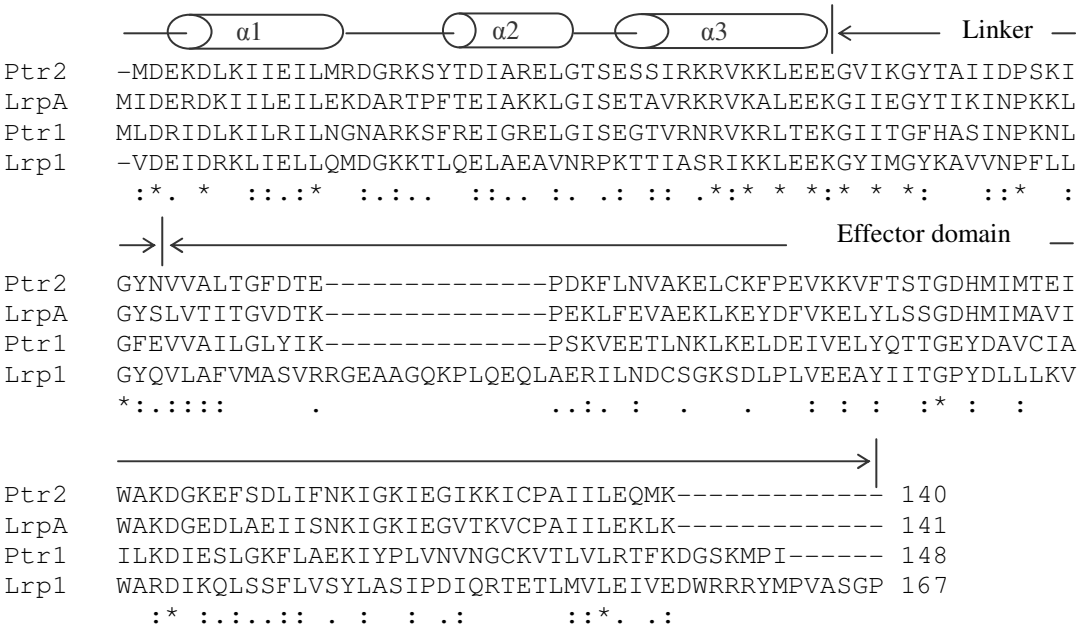
LysM from *S. solfataricus* induces the lysine biosynthesis gene operon (*lysWKJK*; Brinkmann *et al.*, 2002) and Ptr2 from *M. jannaschii* activates transcription of the *fdxA* gene encoding ferredoxin by recruitment of TBP (Ouhammouch *et al.*, 2003).

In Bacteria, Lrp is involved in transcriptional regulation of many different genes, e.g. mainly involved in amino acid or nitrogen metabolism and peptide transport and therefore, rather represents a global regulator. The *E. coli* Lrp represents the best studied member of the Lrp family (Calvo and Matthews, 1994; Newman and Lin, 1995) and microarray studies revealed, that transcription of at least 10% of all *E. coli* genes is affected by Lrp in stationary growth phase (Tani *et al.*, 2002).

The leucine-responsive regulator protein (Lrp1) of T. tenax

In the genome of *T. tenax* at all seven ORFs (TTX_0100, TTX_0884, TTX_1152, TTX_1154, TTX_1497, TTX_1498, TTX_2087) encode putative transcriptional regulators, belonging to the Lrp/AsnC family (COG1522).
First functional analyses of the recombinant, putative leucine-responsive regulator protein Lrp1 (TTX_1154) and the conserved hypothetical protein HP5 (TTX_1155) of *T. tenax* were performed using electrophoretic mobility shift assays (EMSAs) in order to investigate their DNA-binding capacity to their own promoter region.
The organisation of both genes, which are located upstream of the ED gene cluster (see fig. 3.10), indicated a possible functional relation to the transcription of the ED genes. To confirm an involvement of the putative Lrp1 as well as HP5 in the transcriptional regulation of the ED gene cluster, comprising the genes coding for gluconate dehydratase, KD(P)G aldolase, KDG kinase and glucoamylase of *T. tenax*, the binding studies were also performed using a DNA fragment spanning the promoter region of the ED gene cluster (see 2.4.11 and 3.2.3).

The *T. tenax* Lrp1 shares significant sequence similarity with the characterised LrpA from *P. furiosus* (25% aa identity; PF1601), which is shown to negatively regulate its own transcription by abrogation of RNAP recruitment (Brinkmann *et al.*, 2000; Dahlke and Thomm, 2002) as well as with Ptr1 and Ptr2 from *M. jannaschii* (26% and 28% identity, respectively; MJ0151 and MJ0723; Ouhammouch and Geiduschek, 2001; see fig. 4.2), and with other archaeal putative transcriptional regulators of the Lrp/AsnC family from other Archaea, e.g. *P. aerophilum* (70% identity, PAE3471), *Thermococcus kodakaraensis* (35% aa identity TK1210) or *Pyrococcus abyssi* (36% aa identity, PAB6490). The N-terminal domain of the *T. tenax* Lrp1 contains a helix-turn-helix fold predicted by NPS@ (Network Protein Sequence Analysis; Combet *et al.*, 2000).
ORF TTX_1155 (HP5) only shows homology to conserved hypothetical proteins from, e.g. *P. aerophilum* (76% aa identity; PAE3481), *P. islandicum* (73% aa identity; Pisl0777) or *Thermofilum pendens* (45% aa identity; Tpen0107). For HP5 no conserved domains performing CD search at NCBI (Marchler-Bauer *et al.*, 2007) and only less significant HTH motif was predicted by using NPS@. Thus, no characteristic sequence features could be recognised, indicating regulatory properties of this protein. Nevertheless, also this protein was checked for specific DNA binding capacity.

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Ptr2   -MDEKDLKIIEILMRDGRKSYTDIARELGTSESSIRKRVKKLEEEGVIKGYTAIIDPSKI 59
LrpA   MIDERDKIILEILEKDARTPFTEIAKKLGISETAVRKRVKALEEKGIIEGYTIKINPKKL 60
Ptr1   MLDRIDLKILRILNGNARKSFREIGRELGISEGTVRNRVKRLTEKGIITGFHASINPKNL 60
Lrp1   -VDEIDRKLIELLQMDGKKTLQELAEAVNRPKTTIASRIKKLEEKGYIMGYKAVVNPFLL 59
        :*. *  ::.:*  :.:..  ::.. :. .: :: .*:* * *:* * *:   ::*  :

Ptr2   GYNVVALTGFDTE--------------PDKFLNVAKELCKFPEVKKVFTSTGDHMIMTEI 105
LrpA   GYSLVTITGVDTK--------------PEKLFEVAEKLKEYDFVKELYLSSGDHMIMAVI 106
Ptr1   GFEVVAILGLYIK--------------PSKVEETLNKLKELDEIVELYQTTGEYDAVCIA 106
Lrp1   GYQVLAFVMASVRRGEAAGQKPLQEQLAERILNDCSGKSDLPLVEEAYIITGPYDLLLKV 119
       *:.::::     .              ..:. :  .   .   : : :  :* :  :

Ptr2   WAKDGKEFSDLIFNKIGKIEGIKKICPAIILEQMK------------- 140
LrpA   WAKDGEDLAEIISNKIGKIEGVTKVCPAIILEKLK------------- 141
Ptr1   ILKDIESLGKFLAEKIYPLVNVNGCKVTLVLRTFKDGSKMPI------ 148
Lrp1   WARDIKQLSSFLVSYLASIPDIQRTETLMVLEIVEDWRRRYMPVASGP 167
         :* :.:..:: . :  : .:      ::*. .:
```

Fig. 4.2 **Multiple sequence alignment of *T. tenax* Lrp1, *P. furiosus* LrpA, *M. jannaschii* Ptr1 and Ptr2.** The alignment was performed with CLUSTAL W (1.83). Conserved (*****) and chemically similar (**.** and **:**) amino acid residues are marked. The putative DNA-binding (HTH motif), linker and effector domains are indicated above the alignment (predictions were performed by NPS@, CD search NCBI and APSSP2)

Heterlogous expression of the Lrp1 revealed three protein species with a molecular mass of about 18-20 kDa as deduced from SDS-PAGE corresponding approximately to the size generally observed for Lrp proteins. Recombinant expression of HP5 resulted in a single protein species a molecular mass of 15 kDa as deduced from SDS-PAGE.

EMSAs performed with the recombinant Lrp1 and HP5 of *T. tenax* using PCR-amplified fragments spanning their own promoter regions as well as of the ED genes were used as target sequences (see fig. 3.8). Lrp1 was shown to specifically bind to its own promoter and therefore a negatively regulation of its transcription is proposed, like it is described for, e.g. LrpA from *P. furiosus* (Brinkmann *et al.*, 2000; Dahlke and Thomm, 2002). However, HP5 showed no DNA-binding ability.

Furthermore, instable binding of Lrp1 to the promoter region of the ED gene cluster was found suggesting a possible function in the transcriptional regulation of the ED genes.

For several of the characterised archaeal Lrp homologs, e.g. the Ss-LrpB from *S. solfataricus* (SSO2131; She *et al.*, 2001) as well as for the Ptr1 and Ptr2 from *M. jannaschii* (28% aa identity to *T. tenax* MJ0151 and MJ0723; Bult *et al.*, 1996) binding sites and respective consensus sequences are identified (see fig. 4.3). These consensus binding sequences differ, even those of Ptr1 and Ptr2, however, they share two general features: the consensus

represents a palindrome (imperfect for *E. coli*) and the sequences are A-T rich (at least at the dyad).

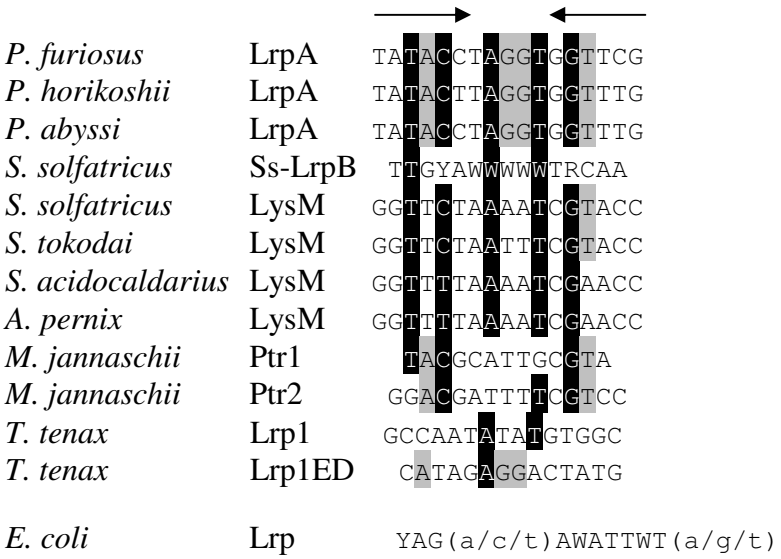


Fig. 4.3 **Binding sites of archaeal Lrp homologs and *E. coli* Lrp.** Binding sites from *M. jannaschii* Ptr1, Ptr2 identified by Ouhammouch and Geiduschek, 2001; *P. furiosus* LrpA, *S. solfataricus* LysM by Brinkman *et al.*, 2000 and 2002; *E. coli* Lrp by Cui *et al.*, 1995; remaining (except *T. tenax* Lrp1 and Lrp1ED) predicted by Brinkmann *et al.*, 2003. Horizontal arrows indicate (partial) inverted repeats. Capital letters consensus: less mismatch propability (than small letters), w/W: A or T, r/R: purine, y/Y: pyrimidine.

In the promoter region of the *T. tenax lrp1* a putative binding site was found (Lrp1; 123 bp upstream of putative start; see fig. 4.3) that shows the proposed features of the so far identified consensus sequences (palindromic, AT-rich) and a corresponding length of 15 bp. The upstream regions of the ED genes were also checked for a respective motif and 107 bp upstream of the transcription start site of the *kdgA-kdgK-gaa* operon a 14 bp long sequence was found (Lrp1ED, see fig. 4.3) that may represents a putative Lrp1 binding site.

For some archaeal Lrp homologs, e.g. Lrs14 and Ss-LrpB from *S. solfataricus* (Napoli *et al.*, 1999; Peeters *et al.*, 2004) as well as Sa-Lrp from *Sulfolobus acidocaldarius* (Enoru-Eta *et al.*, 2000) it was shown that they bind to multiple sites (2 to 3) in their promoter region.

From the binding studies it can be concluded that the *T. tenax* Lrp1 plays a possible role in the regulation of the ED genes. However, the microarray analyses revealed no significant differential gene expression of the respective genes comprised in the cluster, comparing autotrophic versus heterotrophic growth of *T. tenax*. Obviously, the ED genes are regulated by different growth conditions (see 4.1.2). Due to the slight induction of the *ilvD-garK* gene

cluster coding for the dihydroxy-acid dehydratase (DHAD) and glycerate kinase (GK), key enzyme of the non-phosphorylated ED pathway, in CO_2 grown cells, a possible additional role of the ED pathway in amino acid metabolism is discussed (see 4.1.2).

To really proof negative autoregulation and particularly positive or negative regulation of the ED genes by Lrp1 as well as to analyse effector interactions, it would be necessary to apply Lrp1 to a cell-free *in vitro* transcription system, which is currently set up for *T. tenax* (like it has already been described for other Archaea, e.g. *P. furiosus* (Hethke *et al.*, 1996)). Furthermore, e.g. footprint analyses are needed for an exact determination of the Lrp1 binding site. Future analyses imply the whole genome exploration of the Lrp1 regulon that can be performed by determination of the binding sites either *in vitro* using runoff transcription/macroarray analyses (ROMA; Cao *et al.*, 2002) or *in vivo* performing chromatin immunoprecipitation (ChIp) analyses (Shannon and Rao, 2002).

As mentioned previously, the genome of *T. tenax* harbours seven Lrp homologs (COG1522). Strikingly, one of these Lrp candidates (Lrp2, TTX_1152) is located downstream of Lrp1 (see fig. 4.4). A quite similar organisation of respective homologs, are found in the closely related *Pyrobaculum* species (*P. aerophilum, P. islandicum, P. calidifontis*).

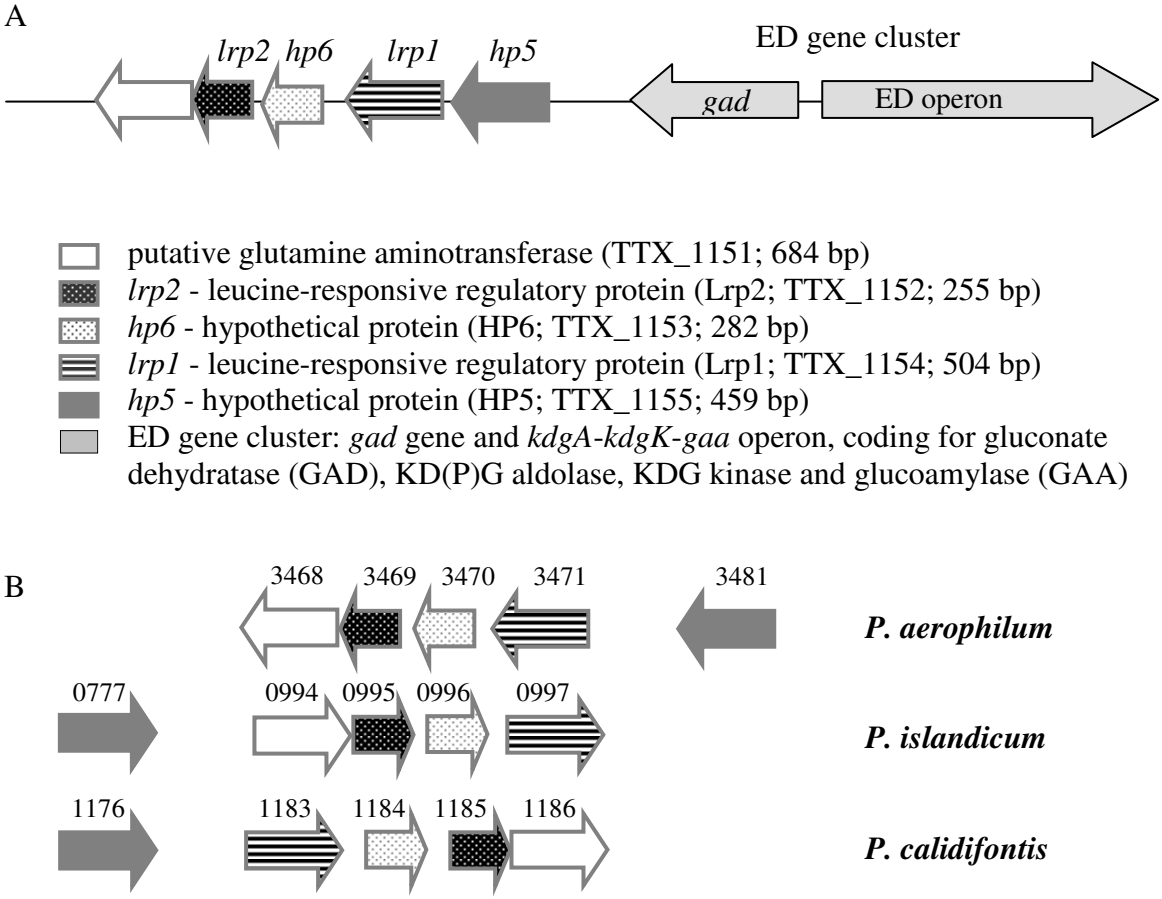


Fig. 4.4 **Clustering of the ED genes with putative transcriptional regulators.** Genes and their orientation are shown as arrows. The key for genes is given below. **A)** The *lrp1* and *hp6* gene are separated by 55 bp, *hp6* and *lrp2* by 31 bp and the latter overlaps by 1 bp with a putative glutamine aminotransferase. **B)** A conserved genome organisation is found within *Pyrobaculum sp.*

Lrp2 encoded by TTX_1152 (255 bp) represents a smaller Lrp-like protein (85 aa), than Lrp1 as well as the characterised *P. furiosus* LrpA and *M. jannaschii* Ptr1 and Ptr2 and it shares less similarity. A HTH motif (with less significance) is predicted by @NPS. However, Lrp2 shares high similarity with several other Lrp, which also seem to be reduced in size, from *Pyrobaculum sp.* (67%, 66% and 63% aa identity with *P. calidifontis, P. aerophilum, P. islandicum*), *S. tokodaii* and *S. acidoacaldarius* (45% aa identity), *A. pernix* (43% aa identity), *Thermofilum pendens* (42% aa identity) or *P. furiosus* (40% aa identity), may indicating another, novel group of Lrp-like proteins in Archaea.

Strinkingly, homologs of HP6, which is located upstream of Lrp2, were only found in the three *Pyrobaculum* species (see fig. 4.4). Thus, the genomic orgainsation of *lrp1-hp6- lrp2* seems to be restricted to the *Thermoproteaceae*.

4.4 Stress adaptation in *T. tenax*: Investigations of the trehalose metabolism

The nonreducing disaccharide trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is widely distributed in Bacteria, Eucarya and Archaea. Beside an initially supposed function as carbon and energy source (Elbein, 1974) meanwhile, a predominant role of trehalose was shown in protection of proteins and cellular membranes from inactivation or denaturation caused by a variety of stress conditions, including temperature (heat, cold), oxidation, osmolarity, dehydration or dessication (Crowe *et al.*, 1984; Hottiger *et al.*, 1987; De Virgilio *et al.*, 1994; Giaever *et al.*, 1988; Strom and Kaasen *et al.*, 1993). Furthermore, trehalose is supposed to serve as a signalling molecule to direct control certain metabolic pathways and even to affect growth and development in yeast and plants, e.g. *Arabidopsis thaliana* (Blazquez *et al.*, 1993; Thevelein, 1992; Hohmann *et al.*, 1993; Vogel *et al.*, 1998; Elbein *et al.*, 2003).
Trehalose accumulation has been reported in several members of the Archaea (Nicolaus *et al.*, 1988; Martins *et al.*, 1996), however the respective synthesis pathways are not studied in more detail and the function of the disaccharide in Archaea is still unknown.

Several pathways of trehalose synthesis have been described (see introduction and fig. 1.6), however, so far only alternative pathways of the common bacterial and eucaryal OtsA/OtsB (TPS/TPP) pathway have been described in Archaea, e.g. the TreY-TreZ pathway (see fig. 1.6) of the *Sulfolobales* (*S. solfataricus*, *S. acidocaldarius*, *S. shibatae*; Maruta *et al.*, 1996; Kobayashi *et al.*, 1996; Di Lernia *et al.*, 1998).
The identification of one gene homolog in the genome of *T. tenax*, coding for the trehalose-6-phosphate synthase/phosphatase (TPSP), implied the existence of the bacterial and eucaryal OtsA/OtsB pathway in Archaea. First functional analyses of the separated TPS and TPP domain revealed respective TPS and TPP activity, approving an active OtsA/OtsB pathway in *T. tenax* (Brenner, 2001; Zaparty, 2003; Siebers *et al.*, 2004).

The *tpsp* gene (TTX_1304) is organised in an operon with the two upstream located ORFs TTX_1305 and TTX_1304a, coding for a putative glycosyl transferase (GT) and a putative mechanosensitive channel of small-conductance (see 3.3.1) as shown by Northern blot analyses (Zaparty, 2003). This organisation of the three genes in the *msc-gt-tpsp* operon, indicated a function-related association of the encoded proteins. To confirm a functional relation, biochemical studies with the recombinant proteins were performed.

Therefore, the *tpsp*, *gt* and *msc* genes of the *T. tenax* were cloned and heterologously expressed in *E. coli* and *S. solfataricus*, respectively. Recombinant TPSP and GT were functionally analysed due to their enzymatic properties (see 3.3.4 and 4.5.2).
Besides elucidation of the function of trehalose in stress response of *T. tenax*, it is of interest, whether and how the TPSP, the intermediate trehalose 6-phosphate or also trehalose itself, directly or indirectly regulates the different metabolic pathways of the *T. tenax* CCM.

4.4.1 Bifunctional TPSP and putative glycosyl transferase (GT) from *T. tenax*: Enzymatic properties of the recombinant enzymes

The TPSP from *T. tenax* represents a protein (732 aa) composed of two domains: The N-terminal trehalose-6-phosphate synthase (TPS; COG0380, EC 2.4.1.15) and the C-terminal trehalose-6-phosphate phosphatase (TPP; COG1877, EC 3.1.3.12) domain. TPS generally catalyses the formation of trehalose 6-phosphate (Tre6P) from UDP-glucose (UDGP) and glucose 6-phosphate (G6P), TPP dephosphorylates Tre6P to trehalose (OtsA/OtsB pathway). A similar two-domain structure has already been described for TPSP from plants, e.g. *Selaginella lepidophylla*, *Arabidopsis thaliana* (Zentella *et al.*, 1999; Blazquez *et al.*, 1998) and also for *Saccharomyces cerevisiae* (Kaasen *et al.*, 1994). The *T. tenax* TPSP shares high similarity with these proteins (*S. lepidophylla* SlTPS1: 35%, *A. thaliana* AtTPS1: 35% and *S. cerevisiae* ScTPS1: 38% aa identity; see fig. 4.7 and 4.9). However, these TPSP proteins only show either TPS or TPP activity.
In contrast to these proteins, which show only one activity, the present work shows that both domains of the *T. tenax* TPSP are active and thus, the *T. tenax* enzyme represents the first description of a protein with TPSP structure, which possesses both, TPS and TPP activity.

The *tpsp* gene is cotranscribed with ORF TTX_1305 (*gt* gene; see fig. 3.16) coding for a putative glycosyl transferase (GT, 387 aa; EC 2.4.1.-). However, strinkingly, only the C-terminus of the protein shows homology to glycosyl transferase group 1 (pfam00534), e.g. from the Bacteria *Roseiflexus sp.* (25% aa identity, C-terminus) or *Myxococcus xanthus*, DK1622, 27% aa identity, C-terminus). Members of this glycosyl transferase family transfer activated sugars, e.g. UDP or ADP linked sugars, to a variety of substrates including glucose, glycogen or fructose 6-phosphate.

The *T. tenax* GT shares high similarity over the whole sequence length only with putative glycosyl transferases (group 1) from three closely related *Pyrobaculum* species (*P. aerophilum*, PAE1273: 43% aa identity; *P. islandicum*, Pisl1340: 43% aa identity; *P calidifontis*, Pcal1362: 44% aa identity). Homology searches performed only with the N-terminal part (first 200 aa) of the GT resulted only these three homologs.

4.4.1.1 Enzymatic properties of the recombinant TPSP and GT from *T. tenax*

The heterologous expression and SDS-PAGE of the TPSP suspected three protein bands with a molecular mass between 82 to 92 kDA to represent TPSP proteins. N-terminal sequencing analyses revealed identical N-terminal TPSP sequence for these protein species. Initial analyses were performed to elucidate a possible modification of TPSP (phosphorylation) and obtained results do not point to a phosphorylation.

The recombinant GT (~42 kDa) was expressed in inclusion bodies, subsequently purified, refolded from the insoluble aggregates and by this means active recombinant protein was obtained.

The discontinous TPSP and TPSP-GT assays were carried out at 70°C either containing the substrates UDPG and G6P or Tre6P (see 2.8.4 and 3.3.4). Intermediates and products were identified by the rather insensitive TLC (see 2.8.6). To detect lower amounts of product, ^{14}C-labelled G6P was used in addition.

With Tre6P as substrate as substrate, pronounced amounts of trehalose were found, whereas, in the presence of UDPG and G6P only a marginal amount of trehalose could be found followed by the radioactive assay.

These results are confirmed by previous studies of A. Brenner (2001) that revealed only a very low activity of the single TPS domain. Formation of less amount of trehalose 6-phosphate was observed in the presence of UDPG and G6P (at 70°C) monitored by sensitive ion chromatography.

Virtually the same result was observed using ADPG instead of UDPG indicating a rather low substrate specifity of the TPSP. Contrary to that, the two TPS of S*accharomyces* are highly specific for either ADPG or UDPG, respectively (Paschoalin *et al.*, 1989).

In contrast to the single TPS domain, the single TPP domain was shown to be highly active, even at 90°C, forming trehalose from Tre6P (Zaparty, 2003).

Strikingly, noticeable trehalose formation from UDPG or also ADPG and G6P by the bifunctional TPSP was observed in presence of the putative GT (see fig. 3.22).
Since TPS also represents a member of the broad glycosyl transferase family (group 20), and in order to demonstrate that the GT not just substitutes for low TPS activity, trehalose formation was followed in the presence of the GT and the single TPP domain (Zaparty, 2003). No trehalose formation was observed, indicating that either activation via modification of the TPSP (e.g. glycosylation) or protein-protein interaction might be involved.
Alike for the TPSP, it was expected that GT enhances the low activity of the single TPS domain, and that by addition of the single TPP domain (GT + TPS + TPP) trehalose is formed from UDPG and G6P. However, neither Tre6P nor trehalose formation was observed. This may points to an interaction of the GT with the not truncated TPSP to form trehalose from UDPG or ADPG and G6P. GT might be unable to interact with the single domains and therefore no activation of trehalose formation is observed.

For all assays respective controls without protein and substrates, as well as with cell-free extract of *E. coli* were performed. GT and TPSP were also checked for glycosyl-transferring trehalose synthase (TreT) activity. This recently described enzyme from *Thermococcus litoralis* (Qu *et al.*, 2004) catalyses the reversible synthesis of trehalose from UDPG (or ADPG) and glucose. No respective activity was observed for GT and TPSP.

First indications concerning the bifunctional character of the *T. tenax* TPSP were revealed in previous studies, which showed activity of the single TPS and the single TPP domain of the TPSP (Brenner, 2001; Zaparty, 2003). The present study reveals that activity of the bifunctional TPSP is strongly enhanced in presence of the putative GT and therefore confirming the supposed functional relation of the proteins encoded by the trehalose operon (*tpsp-gt-msc*).
Generally, advantages for the organism to employ a bifunctional enzyme may relie on stabilising the intermediates or to increase catalytic efficiency by substrate channelling. Enhanced catalytic efficiency shown for an artificial, bifunctional fusion TPSP constructed of *otsA*/OtsA and *otsB*/OtsB from *E. coli* (Seo *et al.*, 2000), is explained by the direct transfer of trehalose 6-phosphate to the TPP (Seo *et al.*, 2000).

Whether the activation of the TPSP by the GT is due to a modification or an interaction between both enzymes needs to be investigated. Given that no trehalose formation was

observed by cooperation of TPS, TPP and GT strongly points to an interaction of GT and TPSP.

4.4.2 The putative mechanosensitive channel from *T. tenax* (MscTTX)

Mechanosensitive (MS) ion channels represent mechanically gated channels, which respond to changes in membrane tension. Mechanical stimuli that target the cell membrane can widely vary from thermal molecular agitation to potentially destructive cell swelling caused by osmotic pressure gradients (Martinac, 2004).

MS channels have been discovered in organisms belonging to all three domains of life (Sachs, 1988; Morris 1990). Several eucaryal and bacterial MS channels have already been characterised (Colbert *et al.*, 1997; Liedtke *et al.*, 2000; Kim *et al.*, 2003; Sukharev *et al.*, 1994; Chang *et al.*, 1998; Bass *et al.*, 2002). Also four archaeal MS channels have recently been described in *Haloferax volcanii* (MscA1, MscA2; Le Dain *et al.*, 1998), *Thermoplasma acidophilum* (MscTA; Kloda and Martinac, 2001a) and *Methanocaldococcus jannaschii* (MscMJ, MscMJLR; Kloda and Martinac, 2001b,c) that represent bacterial-like rather than eucaryal-like MS channel-type proteins.

Most extensively studied prokaryotic MS channels are the MscL, MscS and MsCM from *E. coli*, which are named due to their conductive properties and pressure needed to activate (L: large, S: small, M: mini; Martinac *et al.*, 1987; Martinac *et al.*, 1992; Zoratti and Ghazi, 1993; Strop *et al.*, 2003).

Bacterial MS channels are postulated to play an essential protective role through regulation of cell volume under osmotic stress conditions (Garcia-Anovernos and Corey, 1997; Sachs and Morris, 1998). Therefore, they function as safety valves of the cell (Levina *et al.*, 1999), which primarily serve to transport osmoticants other than ions under stress relief (Ajouz *et al.*, 1998; Martinac, 2001). The role of MS channels in Archaea has not yet been established, however they are supposed to fulfil similar functions as described for Bacteria.

The ORF located upstream of the *gt* gene in the trehalose operon (see fig. 3.16) shares high similarity with conserved hypothetical proteins of *Pyrobaculum sp.* (*P. aerophilum*, PAE1275:51% aa identity; *P. islandicum*, Pisl1341: 54% aa identity; *P.calidifontis*, Pcal1341: 44% aa identity) and putative MS channels of small conductance (COG0668) from several other Archaea and Bacteria (*Methanosarcina barkeri*, MbarA2984: 27% identity; *M. mazei*,

MM2639: 20% aa identity; *S. solfataricus*, SSO2186: 18% aa identity; *Azotobacter vinelandii*, ZP_00090017: 25% aa identity; *Pseudomonas aeruginosa*, UCBPP-PA14: 24% aa identity).

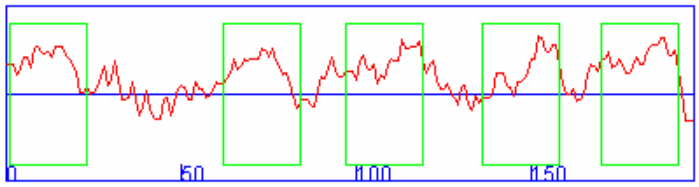
In analogy to the nomenclature used for archaeal MS channels, the putative MS channel of *T. tenax* has been termed MscTTX.

The homology searches with MscTTX revealed no significant similarity with one of the already described archaeal MS channels (see above) that all are characterised to be of large conductance and in contrast to MscTTX show significant similarity to the MscL of *E. coli*.

In many other archaeal genomes MscTTX homologs were identified, indicating a widely distribution and high diversity of MS channels within the Archaea.

However, MscTTX shares a common feature with MscMJ and MscMJLR from *M. jannaschii* represented by five putative hydrophobic transmembrane regions (TM1-5) predicted by TMHMM and SOSUI. Figure 4.5 (A) shows hydropathy plot analysis combined with secondary structure prediction.

A



B

```
  1  gggcccaacatggactcttggaatacctcatcgtcctgtctgtc
  1   G  P  N  M  G  L  L  E  Y  L  I  V  L  S  V

 46  ggcgctctcatcatgttgttgggctatgcggccggcaggctggcc
 16   G  A  L  I  M  L  L  G  Y  A  A  G  R  L  A

 91  aagttccttctctcggagatattcagaaggctcgggctcaacgac
 31   K  F  L  L  S  E  I  F  R  R  L  G  L  N  D

136  tggttcagaaacttcaacatagggagggctatgttgaagtccggc
 46   W  F  R  N  F  N  I  G  R  A  M  L  K  S  G

181  ttcaccgccggggacttcttcgcctctctggccgcttgggtgatc
 61   F  T  A  G  D  F  F  A  S  L  A  A  W  V  I

226  tatatcgccgccttccttgcggcgggctcctatgtgtcgaacaac
 76   Y  I  A  A  F  L  A  A  G  S  Y  V  S  N  N

271  ttcggctacgcctatgtggcggccctcttcgacgaagtcttggcc
 91   F  G  Y  A  Y  V  A  A  L  F  D  E  V  L  A

316  gtgtacgtctacggctttgtgaagttcttcgtggcggccatagtg
106   V  Y  V  Y  G  F  V  K  F  F  V  A  A  I  V

361  ggctttataatggtggacggcttcgtggagtacgtctacaagggc
121   G  F  I  M  V  D  G  F  V  E  Y  V  Y  K  G

406  gctgtctcaaaggaggcggagctcgtgggacctatagcggactac
136   A  V  S  K  E  A  E  L  V  G  P  I  A  D  Y

451  ctcaggatagtgctctacctcgtggtggtcaccttcgccctccaa
151   L  R  I  V  L  Y  L  V  V  V  T  F  A  L  Q

496  cagggcggcatagacgtctcgatactgagcgccatgcttatgcct
166   Q  G  G  I  D  V  S  I  L  S  A  M  L  M  P

541  atcgcgtggggcctggtcgcggccatggtcgccgtcgtgatcgcc
181   I  A  W  G  L  V  A  A  M  V  A  V  V  I  A

586  cggctcctcaaaagatgaacgtagctgtagtggcgccgcagacct
196   R  L  L  K  R  *  T  *  L  *  W  R  R  R  P
```

TM1 TM2 TM3 TM4 TM5

No.	N-terminal	TM region	C-terminal	length
1	1	MGLLEYLIVLSVGALIMLLGYAA	23	23
2	62	DFFASLAAWVIYIAAFLAAGSYV	84	23
3	97	FDEVLAVYVYGFVKFFVAAIVGF	119	23
4	136	KEAELVGPIADYLRIVLYLVVVT	158	23
5	170	ILSAMLMPIAWGLVAAMVAVVIA	192	23

Fig. 4.5 **Putative structural properties of MscTTX. A)** Hydropathy profile revealing five transmembrane (TM1-5) segments and predicted α-helical secondary structures **B)** MscTTX nucleic acid and deduced amino acid sequence. Start and stop codon (bold), TM1-5 (shaded),

table gives position and length of TM segments. Predictions were performed by using SOSUI and TMHMM.

The heterologous expression of MscTTX resulted to be toxic for *E. coli*. A similar effect on *E. coli* growth was described for the expression of MscMJ from *M. jannaschii* (Kloda and Martinac, 2001b), although *E. coli* harbours three Msc (MscL, MscS, MscM) in its membrane. Different MS channels require various levels of cellular turgor to be activated (Martinac, 2000) and therefore, MscTTX might be frequently more open in *E. coli*, due to a higher cellular turgor of *E. coli* relative to the extracellular environment than in *T. tenax*, thus leading to an enhanced leaking of the *E. coli* cells.
Using the *E. coli* strain BL21(DE3) containing pLys and by increasing the osmolarity of the medium, growth of the host was partially improved (see 3.3.6). However, no recombinant protein was obtained. Finally, MscTTX was expressed in the hyperthermophilic Crenarchaeum *S. solfataricus*. MscTTX did not affect growth of *S. solfataricus* as described for *E. coli.* Strikingly, MscTTX shares significant similarity to a hypothetical protein of *S. solfataricus* (SSO2168, 18% identity) possibly encoding *S. solfataricus* MS channel, indicating a similar type of MS channels in the two archaeal species. Expression, solubilisation from the membrane and purification was performed by Dr. S.V. Albers (University of Groningen (NL)).
Given that the effect on growth of *E. coli* could be partially rescued in media with higher osmolarity and the predicted structural features (see fig. 4.5) strongly indicate that the putative MscTTX truly represents a membrane MS channel of *T. tenax*. However, functional analysis has to be performed in order to proof conductive properties of the protein.

4.4.3 Genomic context analysis in Archaea

Using a comparative genomics approach the genes coding for the OtsA/OtsB pathway were identified in different Archaea (see fig.4.6), revealing the presence of fused *tpsp* as well as separated *tps* and *tpp* genes.
The genome organisation of the closely related *Pyrobaculum* species, *P. aerophilum, P. calidifontis* and *P. islandicum* is quite similar to that of *T. tenax* (*tpsp-gt-msc* operon).
In contrast to *T. tenax* and *Pyrobaculum sp.*, which possess a *tpsp*-gene, the genes responsible for trehalose-synthesis are either separated or form an operon in other Archaea (see fig.4.6), e.g. in *Methanothermobacter thermautotrophicus* the *tps* and *tpp* gene are separated by genes

involved in the synthesis of mannosylglycerate (Martins *et al.*, 1999), another compatible solute common in Archaea (Martins *et al.*, 1996). In the genome of *Thermoplasma acidophilum*, *Thermoplasma volcanium* and *Ferroplasma acidarmanus* the ORFs coding for *tps* and *tpp* overlap and seem to form an operon. Therefore, so far, TPSP structure is only found in *T. tenax* and *Pyrobaculum* species, whereas in other Archaea gene organisation resembles that of *E. coli* (single *otsA/tps* and *otsB/tpp* genes).

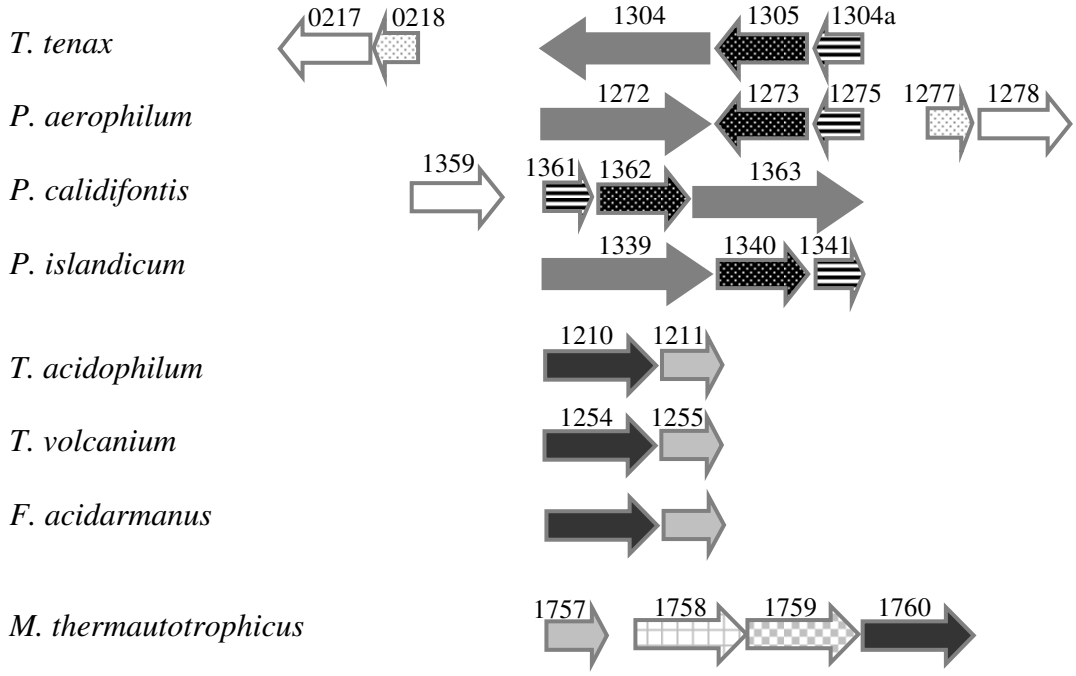


Fig. 4.6 **Comparative genomics with the *tpsp* gene of *T. tenax* arised the presence of the OtsA/OtsB pathway in different Archaea.** Genomic context analyses were performed using LBMGE Genomics ToolBox, UCSC Archaeal Genome Browser and IMG 2.0 (see 2.5.11). Genes and their orientation are shown as arrows. The key for genes is given below.

Trehalose accumulation was concordantly observed in *T. acidophilum* (0,5 µmol/mg protein), *T. tenax* (0,3 µmol/mg proteine) and in *P. aerophilum* (1,1 µmol/mg protein) by Martins *et al.* (1997), where it seems to represent the exclusive compatible solute. *M. thermautotrophicus* was not included in the respective study, but genome data (Smith *et al.,* 1997) suggest the presence of the two solutes mannosylglycerate and trehalose. Furthermore,

genome organisation points a coordinative expression of the gene cluster involved in the synthesis of the two compatible solutes.

Beside the *tpsp* an additional gene homolog (TTX_0217) was identified in the genome of *T. tenax*, which seems to form an operon with an upstream located ORF (TTX_0218). TTX_0217 shares high similarity to the characterised, reversible glycosyl-transferring trehalose synthases (TreT) of *Thermococcus litoralis* (45% aa identity; Qu *et al.*, 2004) and *Pyrococcus horikoshii* (PhGT, PH1035, 45% aa identity; Ryu *et al.*, 2005). Thus, questions arise about a possible role of this gene homolog in trehalose synthesis or degradation. In *P. aerophilum* and *P. calidifontis* the *treT* gene is located directly upstream of the *tpsp-gt-msc* gene cluster, whereas in *T. tenax* the respective ORFs are separated from the trehalose operon (see fig. 4.6).

4.4.4 Structural and phylogenetic aspects of the *T. tenax* TPSP

4.4.4.1 Structural comparison of TPS, TPP and TPSP proteins

Structure of the *T. tenax* TPSP is quite similar to that of TPS2 from *S. cerevisiae* (*Sc*TPS2), which is present as a part of a multienzyme complex consisting of four proteins (see fig. 4.7; Bell *et al.*, 1998; De Virgilio *et al.*, 1993): *Sc*TPS1 represents TPS, *Sc*TPS2 shows TPP activity, whereas TPS3 and TSL1 neither possess TPS nor TPP activity. Both are supposed to fulfil regulatory functions (Londesborough and Vuorio, 1991; Vuorio, 1993; Bell *et al.*, 1998). TPS2, TPS3 and TSL1 all show TPSP structure (see fig. 4.7). So far, this complex is only described for yeast. A regulatory function on trehalose metabolism, including substrate channelling, as well as on glycolysis and fermentation is discussed (Bell *et al.*, 1992 and 1998; Noubhani *et al.*, 2000).

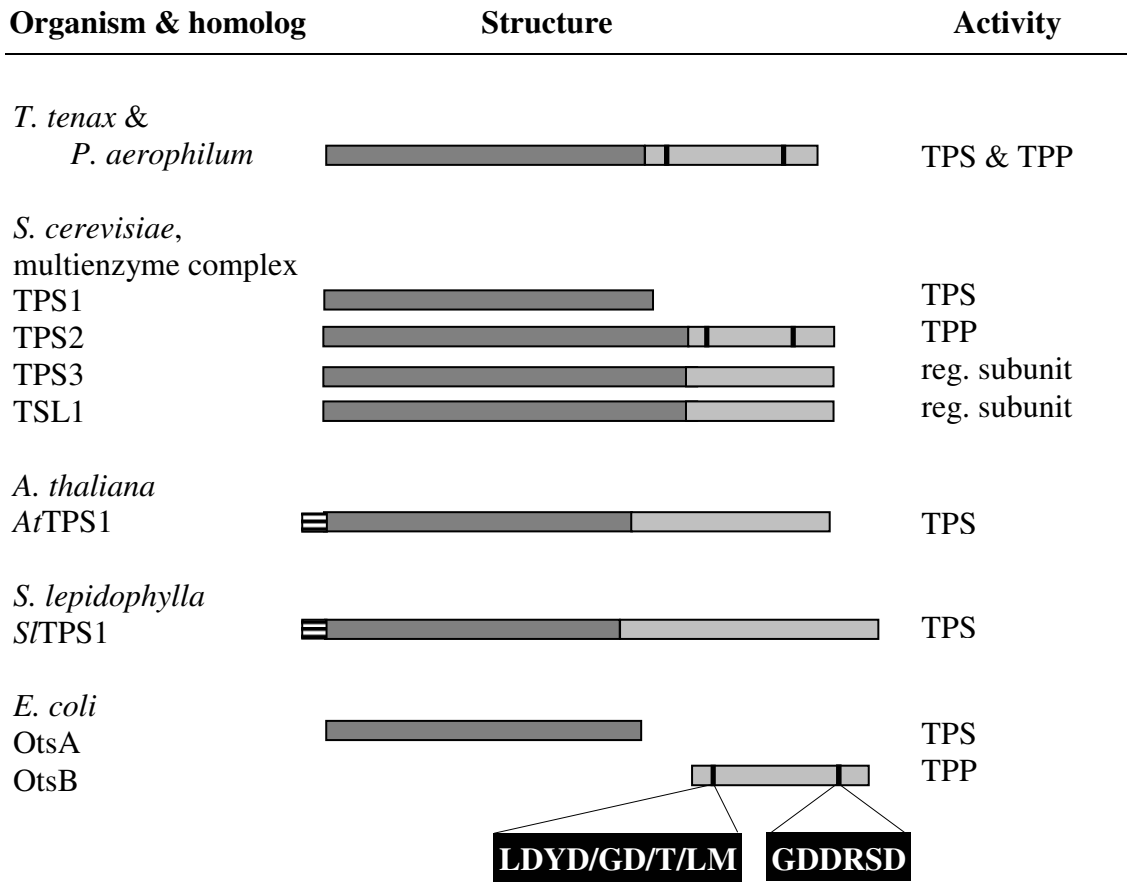


Fig. 4.7 **Structural and functional comparison of TPSP, TPS and TPP from Archaea, Bacteria and Eucarya.** The structure of the fused TPSP consisting of the TPS domain (dark) and the TPP domain (light grey) as well as the single TPS and TPP are shown. The determined enzyme activities are given on the right. Well conserved sequence motifs of active TPP (black bars, boxes) and N-terminal extension of *At*TPS1 and *Sl*TPS1 (striped) are indicated.

The similar structures of *T. tenax* TPSP and *Sc*TPS2 is also reflected in the N- and C-terminal sequence of the TPP domain, where specific sequence motifs are found: -LDYD/GD/T/LM- (N-terminus) and -GDDRSD- (C-terminus) that are supposed to be specific for active TPP domains (see fig. 4.7; VOGEL *et al.*, 1998). Correspondingly, *Sc*TPS3, *Sc*TSL1 and *Sl*TPS1 (Zentella *et al.*, 1999) as well as *At*TPS1 (Blazquez *et al.*, 1998; Vogel *et al.*, 1998) lack these motifs. However, recently similar conserved motifs were also identified in TPS domains of *A. thaliana* TPSP homologs, which neither possess TPS nor TPP activity (Vogel *et al.*, 2001).

No homologs of the yeast *Sc*TPS3 and *Sc*TSL1 regulatory subunits were identified in plants. However, for plants a N-terminal extension of about 100 amino acid residues, which shares sequence homology to parts of *Sc*TSL1, is supposed to take over regulatory function (Leyman

et al., 2001). Truncation of this N-terminal sequence extension leads to increased TPS activitiy (Van Dijck *et al.*, 2002).
In *T. tenax* neither homologs of *Sc*TPS3 and *Sc*TSL1 are identified so far, nor does the TPSP contain a regulatory N-terminal extension similar to that of *A. thaliana* (Zaparty, 2003) and therefore, does not explain the low TPS activity of TPSP (see 4.4.1.1).
These findings are also true for Bacteria and fungi, indicating that in these organisms other regulatory features are responsible for the regulation of trehalose metabolism.

4.4.1.2 Phylogenetic aspects

The comparison of TPS and TPP reveals a high similarity between the two domains suggesting an early gene duplication that lead to both enzyme activities. Later fusion events conserved both activities or lost either one or both, as shown for the regulatory subunits of *S. cerevisiae* (Bell *et al.*, 1998) or *A. thaliana* (Leyman *et al.*, 2001; van Dijck *et al.*, 2002). Emergence of the TPSP multienzyme complex from *S. cerevisiae* is also referred to gene duplication or gene fusion events in the evolution of *tps* and *tpp* genes (Kaasen *et al.*, 1994).
This evolution of the different structures and enzyme activities is also reflected in the phylogenetic tree (see fig. 4.8), which shows a clear clustering of proteins with TPS, TPP or TPSP activity in Bacteria and Archaea. The eucaryal proteins (with TPSP structure) show a clear separation or clustering according to their enzyme activity.
Due to the close clustering of the *T. tenax* TPSP with the only archaeal TPSP of *P. aerophilum* and that of *Cytophaga hutchinsonii* (40% aa identity), it is tempting to speculate if these two TPSP proteins also represent bifunctional enzymes, like the *T. tenax* enzyme.
Figure 4.9 shows a multiple sequence alignment of the *T. tenax* TPSP with five homologs showing TPSP structure that represent selected members of each branch of the phylogenetic tree.

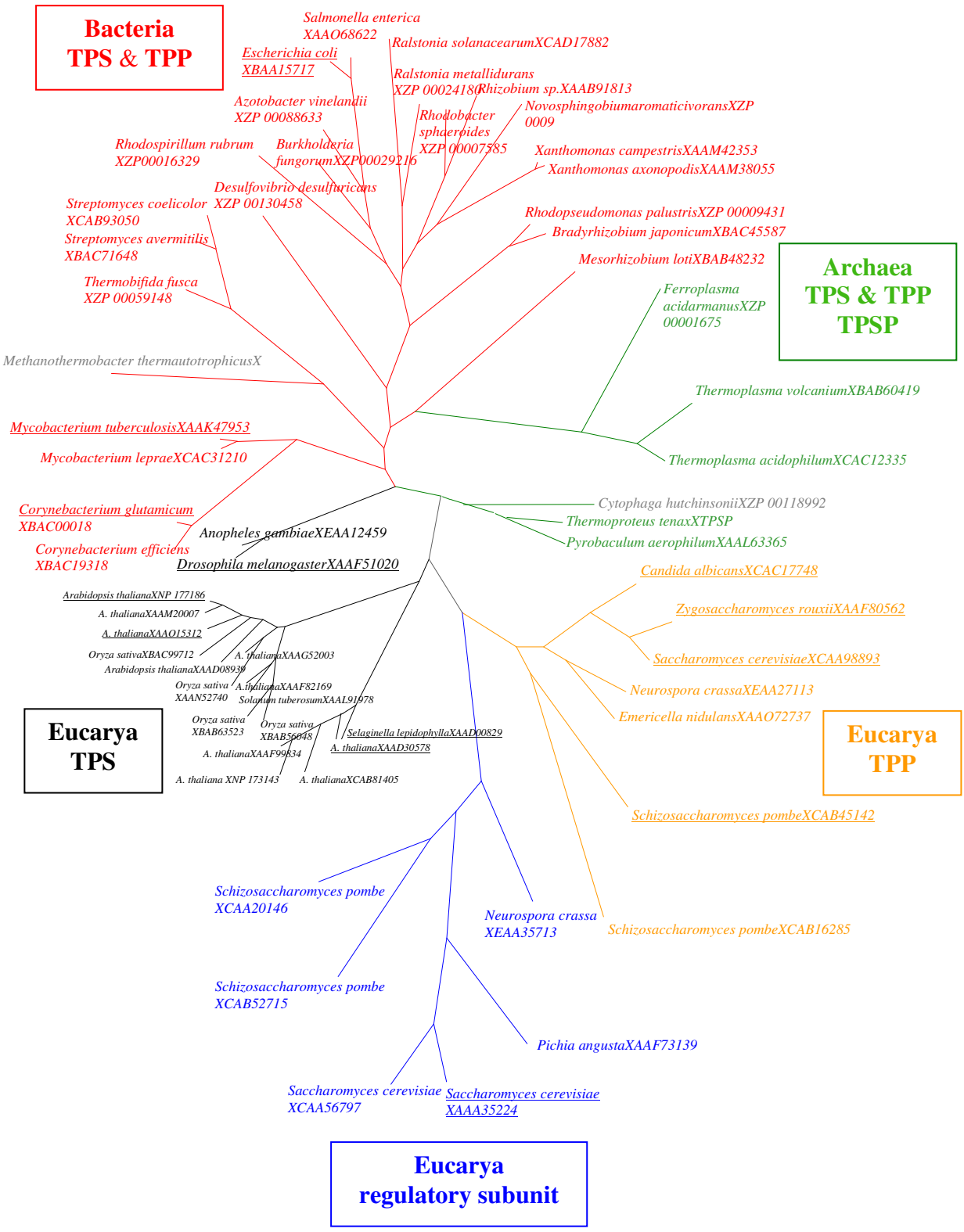


Fig. 4.8 **Phylogenetic tree of *T. tenax* TPSP homologs.** Homology searches were performed using BLAST analyses (Altschul *et al.*, 1990). Alignments and the phylogenetic tree were generated by using CLUSTAL W (1.83; Thompson *et al.*, 1994). This work was performed by Dr. H. Brinkman (University of Montreal (Canada)). **Red cluster**: Bacterial branch of TPS and TPP proteins, with the only exception of *M. thermautotrophicus*. **Green cluster**: Archaeal TPS, TPP and the two TPSP proteins of *T. tenax* and *P. aerophilum*, with the only

exception of TPSP homolog from *Cytophaga hutchinsonii*. **Black, blue and yellow clusters**: Three eucaryal branches of TPSP proteins that either possess TPS (black), TPP (yellow) or regulatory (blue) function. Characterised homologs of the different clusters are underlined.

```
TTX    ------------------------------------------------------------
PAE    ------------------------------------------------------------
SlTPS1 ------------------------------------------------------------
AtTPS1 ------------------------------------------------------------
ScTPS2 ------------------------------------------------------------
ScTPS3 MTIIVASLFLPYTPQFEADVTNSDTAKLVESSMIKVDCNNQELSNNKQERSSSVTSASSH

TTX    ------------------------------------------------------------
PAE    ------------------------------------------------------------
SlTPS1 ------------------------------------------------------------
AtTPS1 ------------------------------------------------------------
ScTPS2 ------------------------------------------------------------
ScTPS3 YIGLPQEAQINGEPLQRANVGSPATGVNYHNEMEMLSSEQFLEELTAnATHAANSGIPPA

TTX     -----------------------------------------------------------
PAE    ------------------------------------------------------------
SlTPS1 ----------------------MPQPYPSSSSTSNAKEAGGGAAAAGGGGGGAFSLPPS
AtTPS1  ------------------------MPGNK--YNCSSS---------------HIP--
ScTPS2  -----------------------------------------------------------
ScTPS3 NNPVSSGSTAQRPSVEEFFSAPSARVCSPSQEASASSISASRSSAHHNDLSSSLMKNPNL

TTX    ------------------------------------------------------------
PAE    ------------------------------------------------------------
SlTPS1 LASSRVERLVRE-------------------------RQLRNQRQED---EP-------
AtTPS1 --LSRTERLLRD-----------------------RELREKRKSNRARNPNDVAGSS
ScTPS2 ----------------------------------MTTTAQDNSPKKRQR------I
ScTPS3 SFDSHPPRVRSSSKSAVITPVSKSVPDVDPAVVDVAKVREEFQQQASLPSMKRVSGSTAG

TTX    ---------------------------------------------VRLIVVSNRLPVTIS
PAE    ---------------------------------------------MRLIIVSNRLPVVLT
SlTPS1 EDEQQ--ALEAEEAAVAATEVPDAVAAATPSLSD-EPSKISSGRGQRLLVVANRLPLSAT
AtTPS1 ENSENDLRLEGDSSRQYVEQYLEGAAAAMAHDDACERQEVRPYNRQRLLVVANRLPVSAV
ScTPS2 INCVTQLPYKIQLGESNDDWKISATTGNSALFSSLEYLQFDSTEYEQHVVGWTGEITRTE
ScTPS3 DSSIASSSSNLRYSQQFQDNFIEDTDSEDDIDSDLETDATKKYNVPKFGGYSNNAKLRAS
                                                          :     .

TTX    PSG---EIR-ESVGGLATA---MKSFLGAVNGGRELG--LEEVVWVGWSGVPSER---E-
PAE    VGERGMEIR-EAVGGLATA---VKSFIKATENGKALG--FSEVVWAGWSGIKAEQ---E-
SlTPS1 RKG-------ETEWNLEMS---AGGLVSALLGVKQF-----EVTWIGWPGVYVQD---EK
AtTPS1 RRG-------EDSWSLEIS---AGGLVSALLGVKEF-----EARWIGWAGVNVPD---EV
ScTPS2 RNLFTREAK-EKPQDLDDD---PLYLTKEQINGLTT---------TLQDHMKSDK---EA
ScTPS3 LMRNSYELFKHLPWTIVDSDKGNGSLKNAVNIAVAEKTVKEPVSWVGTMGIPTDELPHEV
                .    :         :                          :      *

TTX    -SNDLRERLRGMGLEPVPLSSEEVEGFYEGFSNSTLWPLFH--GFS-----EYATYEEKH
PAE    -SEDLKSRLREMGLLPVSLTAEEVNFFYEGFCNSTLWPLFH--GFT-----VYTVFESKY
SlTPS1 GEKSLRGALEEKGFVPVLLDEATVDQYYNGYCNNVLWPLFHYIGLRQEDRLAATRSLLSQ
AtTPS1 GQKALSKALAEKRCIPVFLDEEIVHQYYNGYCNNILWPLFHYLGLPQEDRLATTRSFQSQ
ScTPS2 KTDTTQTAPVTNNVHPVWLLRK-NQSRWRNYAEKVIWPTFHYILNPS----NEGEQEKNW
ScTPS3 CHKISKKLEQDFSSFPVVTDDITFKGAYKNYAKQILWPTLHY-QIPDNP--NSKAFEDHS
          .           **       .  :..:.:. :** :*

TTX    WRAYRGVNEKYAKAVVALARPGDLVWIHDYHLMLAPAIVREAA---EVGVGFFLHIPFPP
PAE    WEAYVKVNQKYAETVASVANTGDFVWIHDYHLMLMPAMLREMSP--DVSIGFFLHIPFPP
SlTPS1 FNAYKRANRLFAEAVFNFYQEGDVVWCHDYHLMFLPSYLKEKDS--QMKVGWFLHTPFPS
AtTPS1 FAAYKKANQMFADVVNEHYEEGDVVWCHDYHLMFLPKCLKEYNS--KMKVGWFLHTPFPS
ScTPS2 WYDYVKFNEAYAQKIGEVYRKGDIIWIHDYYLLLLPQLLRMKFNDESIIIGYFHHAPWPS
ScTPS3 WDYYQKVNQKFSDRIVSVYKPGDTIWIHDYHLMLVPQMVREKLP--KAKIGFFLHVSFPS
       :  *   *. ::. :    . ** :* ***:*:: *  ::      .  :*:* * .:*.

TTX    AELLQLLPSEWRREILEGLLGSDLVGFHTYEYSANFSRSVVRFLGYK----------VEM
PAE    AEMYQLMPPPWRTALLDGVLASDLVGFHIHEYVNNFVRAVSKFLGYR----------TEA
SlTPS1 SEIYRTLPL--RAELLQGVLAADLVGFHTYDYARHFVSACTRILGLEG---------TPE
AtTPS1 SEIHRTLPS--RSELLRSVLAADLVGFHTYDYARHFVSACTRILGLEG---------TPE
```

```
ScTPS2 NEYFRCLPR--RKQILDGLVGANRICFQNESFSRHFVSSCKRLLDATAKKSKNSSNSDQY
ScTPS3 SEVFRCLAN--RERILEGIIGANFVGFQTKEYKRHFLQTCNRLLAAD----------VSN
        *  : :.   *  :* .::.:: : *:  .:  :*  :  ::*

TTX    GAIAVGHRRVRVGVFPIGIDFDRFYNSSQDPSVVEEMAKLREMLGRAKVVFSIDRLDYTK
PAE    GVIYAGRRKVHVGAFPIGIDFDFFYNSSLDPEVAGQIEELRQKLRGLKIIFSIDRLDYTK
SlTPS1 GVEDQG-KNTRVAAFPVGIDSERFIEAVETDAVKKHMQELSQRFAGRKVMLGVDRLDMIK
AtTPS1 GVEDQG-RLTRVAAFPIGIDSDRFIRALEVPEVIQHMKELKERFAGRKVMLGVDRLDMIK
ScTPS2 QVSVYG-GDVLVDSLPIGVNTTQILKDAFTKDIDSKVLSIKQAYQNKKIIIGRDRLDSVR
ScTPS3 DEVKYHCNIVSVMYAPIGIDYYHLTSQLRNGSVLEWRQLIKERWRNKKLIVCRDQFDRIR
               . *   *:*::   :        :      : :     *::.  *::*  :

TTX    GVLRRVAAWERFLREHPEWRGRAVFVLVVVPSRT-GVPMYEEMKRQIDREVGRINGELGE
PAE    GVINRVHAWERFLKEHPQWRGKASFILIVVPSRI-GVPQYDAMKREIEREVGRINGELGD
SlTPS1 GIPQKLLAFEKFLEENSEWRDKVVLVQIAVPTRT-DVLEYQKLTSQVHEIVGRINGRFGS
AtTPS1 GIPQKILAFEKFLEENANWRDKVVLLQIAVPTRT-DVPEYQKLTSQVHEIVGRINGRFGT
ScTPS2 GVVQKLRAFETFLAMYPEWRDQVVLIQVSSPTANRNSPQTIRLEQQVNELVNSINSEYGN
ScTPS3 GLQKKMLAYERFLIENPEYIEKVVLIQICIGKSS--DP---EYERQIMVVVDRINSLSSN
       *: .:: *:* **   .::  :. :: :   .             ::   *. **.  .

TTX    LNWV-PIVYLYRFIPSPTLMALYNIADVALITPLRDGMNLVAKEFVASK-----RDCRG-
PAE    VNWT-PIVYISRFIPTPTLLALYNIADVALITPLRDGMNLVSKEYVATK-----RDCKG-
SlTPS1 LTAV-PIHHLDRSMKFPELCALYAITDVLLVTSLRDGMNLVSYEFVACQ-----KDKKG-
AtTPS1 LTAV-PIHHLDRSLDFHALCALYAVTDVALVTSLRDGMNLVSYEFVACQ-----EAKKG-
ScTPS2 LNFS-PVQHYYMRIPKDVYLSLLRVADLCLITSVRDGMNTTALEYVTVKSHMSNFLCYGN
ScTPS3 ISISQPVVFLHQDLDFAQYLALNCEADVFLVDALREGMNLTCHEFIVSS------FEKNA
       :.   *: .    :      :*   :*: *: .:*:*** .. *::. .        .

TTX    VLILSELAGASKEL-AEALVINPNDVGGTAEAIAEALSMSEDEQCRRIRAMQERLRMRDV
PAE    VLILSETAGASHEL-LEALIVNPNDESGVVEAIAKALTMEPEEQCRRIKAMQEKLRQQNV
SlTPS1 ALILSEFAGAAQSLGAGSILINPWNIIESSNAIADALNMPEEEREERHRHNFMHITTHSA
AtTPS1 VLILSEFAGAAQSLGAGAILVNPWNITEVAASIGQALNMTAEEREKRHRHNFHHVKTHTA
ScTPS2 PLILSEFSGSSNVL-KDAIVVNPWDSVAVAKSINMALKLDKEEKSNLESKLWKEVP--TI
ScTPS3 PLLLSEFTGSSSVLKEGAILINPWDINHVAQSIKRSLEMSPEEKRRRWKKLFKSVIEHDS
        *:*** :*::  *   ::::** :      :*  :* :  :*: .        :

TTX    VRWGTDFIYSLISAKSAR-EEVEKALRYMEELSVDKLKSDFAKAKRRLLLLDYDGTLVPH
PAE    VKWAVDFLHSLMLAYRENTESFTTSSKLLDREAIEEIVKIFHGARSRLLLLDYDGTLVPH
SlTPS1 QVWAETFISELNDSILEA---ELRTLHIPPQLPLDKAVAKYSESKNRLVILGFNSTLTAQ
AtTPS1 QEWAETFVSELNDTVIEA---QLRISKVPPELPQHDAIQRYSKSNNRLLILGFNATLTEP
ScTPS2 QDWTNKFLSSLKEQASSN---DDMERKMTPALNRPVLLENYKQAKRRLFLFDYDGTLTPI
ScTPS3 DNWITKCFEYINNAWESN-----QETSTVFNLAPEKFCADYKASKKHLFIFKIS------
         *    .  :                                 :  :. :*.::  .

TTX    YPYPHMAVPD----------GDLLELLSRLAALPETAVYVVSGRGR-DFLDGWLG--RLP
PAE    YPYAYQAVPD----------GELKRLLNSLAFQPNTYVAVVSGRGR-DFLEAWLG--DLP
SlTPS1 VEAPRGRAPDQIREMKIRLHPSIKDILNVLCSDPKTTIVILSGSER-VALDEVFG--EFD
AtTPS1 VDN-QGRRGDQIKEMDLNLHPELKGPLKALCSDPSTTIVVLSGSSR-SVLDKNFG--EYD
ScTPS2 VKDPAAAIPS----------ARLYTILQKLCADPHNQIWIISGRDQ-KFLNKWLGGKLPQ
ScTPS3 ------EPPT----------SRMLSLLSELSS--NNIVYVLSSFTKNTFESLYNG--VLN
                          :   *. *.    . : ::*.  :    .   *

TTX    VGLVAEHGFFLKHP-GGEWKSLGK--VDPSWRQYAKGIMEDFASNVPGSFVEVKEAGIAW
PAE    IYIVAEHGAFIRDP-GGNWSQLFP--FDTSWKISVRKIMEEFTRLTPGSYIEEKEISLAW
SlTPS1 LWLAAENGMFLRHT-QGEWMTTMPEHLNMDWLESVQLVFDYFCERTPRSFVETRETSLVW
AtTPS1 MWLAAENGMFLRLT-NGEWMTTMPEHLNMEWVDSVKHVFKYFTERTPRSHFETRDTSLIW
ScTPS2 LGLSAEHGCFMKDVSCQDWVNLTEK-VDMSWQVRVNEVMEEFTTRTPGSFIERKKVALTW
ScTPS3 IGLIAENGAYVRVN--GSWYNIVE---ELDWMKEVAKIFDEKVERLPGSYYKIADSMIRF
       : : **:* :::     .*        : .*   .  ::.      * *. :  .  : :

TTX    HYRNADETIAEKAVVELIDAL-SNALAGSGLSILRGKKVVEVRPAGYTKGTAAKMLLDEL
PAE    HYRNVEPEIGEKAANRLADAL-TGLLESSPANIIRGVKVVEVRAAGVNKGVAAKLLYDKL
SlTPS1 NYKYADVEFGRVQARDMLQHLWTGPISNAAVDVVQGGKSVEVRPVGVSKGSAIDRILGEI
AtTPS1 NYKYADIEFGRLQARDLLQHLWTGPISNASVDVVQGSRSVEVRAVGVTKGAAIDRILGEI
ScTPS2 HYRRTVPELGEFHAKELKEKL-LSFTDDFDLEVMDGKANIEVRPRFVNKGEIVKRLVWHQ
ScTPS3 HTENADDQDRVPTVIGEAITHINTLFDDRDIHAYVHKDIVFVQQTGLA-LAAAEFLMKFY
       : . .        .           .          : *:         . :

TTX    SPDFVFVAGDDE-TDEGMFEVAPQSAYTVKVGPGP----TLAKFRVGDYRGLRSLLEQLR
PAE    RPELVIIAGDDY-TDEEMMKALP-EAITIKVGKGE----TSAKYMAPSYRRIRELLQALL
SlTPS1 VHSKHMTIPIDYVLCIGHFLSKDEDIYTFFEPELP----LLDRDS-STSNGGKP-LGGKL
AtTPS1 VHSKSMTTPIDYVLCIGHFLGKDEDVYTFFEPELPSDMPAIARSRPSSDSGAKSSSGDRR
```

```
ScTPS2 -HGK----PQD--MLKGISEKLPKDEMPDFVLCLG--DDFTDEDMFRQLNTIETCWKEKY
ScTPS3 NSGVS---PTDN-SRISLSRTSSSMSVGNNKKHFQN-QVDFVCVSGSTSPIIEPLFKLVK
                *                                                .

TTX    PP------------------------------------------------------------
PAE    TAQR----------------------------------------------------------
SlTPS1 PIDR------KS-SKSSSRMKPPVSSPKSPGRGSEQQQQAEEASRWEGSS----------
AtTPS1 PPSKSTHNNNKSGSKSSSSSNSNNNN-KSSQR-SLQSERKSGSNHSLGNSRRPSPEKIWS
ScTPS2 PDQK-----NQWGNYGFYPVTVGSASKKTVAKAHLTDPQQVLETLGLLVG----------
ScTPS3 QEVE-------KNNLKFGYTILYGSSRSTYAKEHINGVNELFTILHDLTAA---------

TTX    ------------------------------------------------------------
PAE    ------------------------------------------------------------
SlTPS1 -VLDLQGENYFSCAVGTMKRSLARYCLTSSEEVVTFLTSLTSTVAAAAGAGAGARATGSG


AtTPS1 NVLDLKGENYFSCAVG-RTRTNARYLLGSPDDVVCFLEKLADTTSSPFRMU-LARENDE-
ScTPS2 -HGK----PQD--MLKGISEKLPKDEMPDFVLCLG--DDFTDEDMFRQLNTIETCWKEKY
ScTPS3 ------------------------------------------------------------

TTX    ------------------------------------------------
PAE    ------------------------------------------------
SlTPS1 AAGAGAGAGAGGDHEAPGSPIRKSDSFKTSGWHSPTPRSPKLAPAVQ
AtTPS1 ------------------------------------------------
ScTPS2 ------------------------------------------------
ScTPS3 ------------------------------------------------
```

Fig. 4.9 **Multiple sequence alignment of *T. tenax* TPSP with structural related homologs.** Homology searches were performed using BLAST analyses (Altschul *et al.*, 1990). The alignment was performed with CLUSTAL W (1.83; Thompson *et al.*, 1994). TTX, TPSP from *T. tenax*; PAE, TPSP from *P. aerophilum*; *Sl*TPS1, TPS1 from *S. lepidophylla*; *At*TPS1, TPS from *A. thaliana*; ScTPS2, TPS2 (TPP) and ScTPS3, TPS3 regulatory subunits of *S. cerevisiae*. Identical (*****) and chemically similar (**·** and **:**) amino acid residues are indicated. The TPSP homologs represent selected members of the different branches of the phylogenetic tree (see fig. 4.8, also for accession numbers of the proteins).

4.4.5 Physiological and regulatory role of trehalose in *T. tenax*

An active OtsA/OtsB pathway for trehalose synthesis, so far typical for Bacteria and Eucarya, was identified in *T. tenax*, which additionally is characterised by a novel, bifunctional TPSP protein, whose activity is strongly enhanced in presence of a putative glycosyl transferase.

The *otsA/tps* and *otsB/tpp* genes coding for the enzymes of the OtsA/OtsB pathway could also be identified in other Archaea (see fig. 4.6), revealing the presence of a fused TPSP only in *Pyrobaculum sp.* as well as separated TPS and TPP homologs, like it is described for Bacteria and Eucarya (see fig. 4.6). Despite this distribution of the OtsA/OtsB pathway as well as of alternative synthesis pathways, e.g. TreY-TreZ pathway of the *Sulfolobales*, among the Archaea, the role of trehalose in Archaea still remains unknown. Due to the proven function

of the disaccharide as compatible solute in Bacteria, e.g. accumulation of trehalose in *E. coli* as response to osmotic stress (Giaver *et al.*, 1988; Strom and Kaasen, 1993) and in Eucarya, thermoadaptation of *S. cerevisiae* (Hottiger *et al.*, 987; De Virgilio *et al.*, 1994) a similar function is proposed for the Archaea, several of which are adapted to extreme environments of certain habitats, such as extreme temperatures (heat, cold) or salt concentrations.

Since the disaccaride trehalose is also discussed as a possible carbon and engery source in some organisms, question arises, if this could also be the case in *T. tenax*. Also a double function of trehalose in stress response and as carbon storage, is discussed for some organism, even Archaea (De Virgillio *et al.*, 1990; Martins *et al.*, 1997).

However, some hints point to an exclusive function of trehalose in stress adaptation in *T. tenax*. The importance of trehalose as carbon and energy source withdraws, due to the presence of glycogen, which was shown to represent the carbon storage compound in *T. tenax* (König *et al.*, 1982). Furthermore, in the genome of *T. tenax* no homologs of known trehalose degrading enzymes, e.g. bacterial trehalases could be identified. Otherwise it cannot be excluded that Archaea employ non-homologous enzymes, which are not characterised to date. The function of the identified putative TreT homolog in the genome of *T. tenax* (see fig. 4.6) remains to be shown. The ORF shows high similarity to the characterised TreT from *T. litoralis* and *P. horikoshii* (PhGT), which catalyse the reversible formation of trehalose from ADPG or UDPG and glucose (Qu *et al.*, 2004; Ryu *et al.*, 2005). The *T. litoralis* TreT is supposed to be mainly involved in trehalose degradation due to the clustering with genes of a maltose/trehalose transporter system (Qu *et al.*, 2004). However, the recombinant protein has been shown to favour formation of trehalose.

It was shown that *T. tenax* is unable to grow on trehalose and furthermore, measurements in crude extracts revealed that *T. tenax* seems to be unable to degrade the disaccharide.

These results indicate that trehalose in *T. tenax* might exclusively functions as compatible solute in stress adaptation and therefore, the results of the microarray experiments (see 3.1.5.5) might be explained by less transcript amounts of the respective genes in the unstressed cells grown auto- and heterotrophically, although respective TPSP and GT activities were measured in cell-free extracts of *T. tenax* (see 3.8.5).

The effect of thermal (heat or cold) and osmotic stress will be studied in future experiments, either performing Northern blot or whole genome DNA microarray analysis (global stress response) to further elucidate the function of trehalose in *T. tenax*.

For *Neurospora crassa* it has been described that substrates for trehalose synthesis are provided by glycogen degradation (Noventa-Jordao *et al.*, 1996). A possible role of glycogen metabolism in stress response of *T. tenax* also remains to be shown.

Beside functioning of trehalose as carbon and energy source and in stress adaptation trehalose is supposed to serve as a signalling molecule to, e.g. direct control certain metabolic pathways and even to affect growth in yeast and plants (Blazquez *et al.*, 1993; Thevelein, 1992; Hohmann *et al.*, 1993; Elbein *et al.*, 2003).
As already discussed above, *T. tenax* utilises two pathways for glucose degradation in parallel (modified EMP- and branched ED pathway). However, it was shown that the modified EMP pathway represents the main route for glucose catabolism that was shown to employ a hexokinase with only a very low regulatory potential (see 4.2.1; Dörr *et al.*, 2002). Here the question arises, how the glucose influx into the EMP pathway and therefore also the synthesis of trehalose as well as glycogen is controlled.
Several studies in *S. cerevisiae* (multienzyme complex) revealed that trehalose metabolism has regulatory effect on glycolysis (Noubhani *et al.*, 2000), e.g. due to interaction of TPS with glucose transport and sugar kinase activities (Thevelein, 1992), by preventing an overflow of glycolysis by utilising glucose 6-phosphate (Hohmann *et al.*, 1993), or by the inhibition of hexokinase activity by trehalose 6-phosphate (Bell *et al.*, 1992; Blazquez *et al.*, 1993). The importance of this regulatory function of the TPS on glycolysis in *S. cerevisiae* was demonstrated by a *tps1* yeast deletion mutant that was unable to grow on glucose. Growth was shown to be complemented with *E. coli otsA* (McDougall *et al.*, 1993; Thevelein and Hohmann, 1995). However, for the hexokinase of *T. tenax* no effect of trehalose 6-phosphate on the enzyme activity is observed (Dörr *et al.*, 2002). However, supposably, a regulatory function of the trehalose metabolism in CCM of *T. tenax* cannot be ruled out. For glycogen metabolism of *T. tenax* a regulatory function of the EMP pathway via glucose 1-phosphate, stimulating GAPN is shown (Brunner *et al.*, 1998).

The supposed functional relation of at least the TPSP and GT, belonging to the *T. tenax* trehalose operon (*msc-gt-tpsp*) could be proven by analysing the enzymatic properties of the recombinant proteins. Future studies will focus on the elucidation of the activation mechanism (modification, interaction) of TPSP by the GT.
There are several strong evidences, indicating that MscTTX represents an active MS channel in the membrane of *T. tenax*. A supposed functional relation between TPSP, GT and

MscTTX remains to be proven. Analysis of the conductive properties of MscTTx expressed in *S. solfataricus* will be performed in future studies.

However, due to the organisation and functional relation of genes responsible for trehalose formation together with a gene encoding a putative safety valve of the cell for the adaptation of cellular turgor under stress conditions, the following model of stress response can be discussed for *T. tenax* and maybe also for other hyperthermophiles in general (see fig. 4.10): Under stress conditions, e.g. caused by high osmolarity, trehalose is synthesised via TPSP and GT, resulting in increased intracellular trehalose concentration. In the case of stress relief, the MS channel opens in response to changes in membrane tension resulting in trehalose efflux. The ability to rapidly jettison trehalose, e.g. in response to a quick osmotic downshift, instead of enzymatic degradation, prevents the cell of swelling and bursting. It seems obvious that this stress response might represent a GT-mediated process.

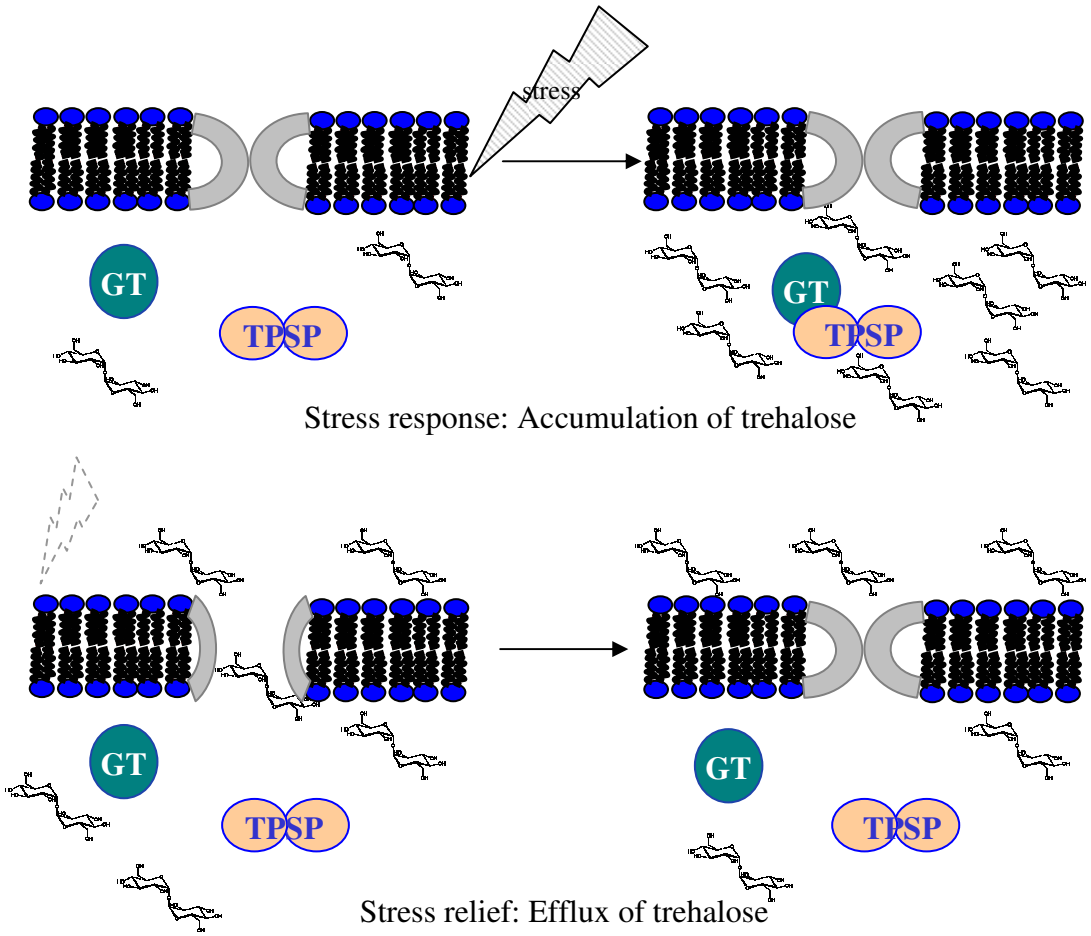


Fig. 4.10 **Proposed model of stress response in *T. tenax***

5 SUMMARY

The metabolic pathways of the *T. tenax* central carbohydrate metabolism (CCM) reflect the complexity and variety of central metabolic pathways that is found as a general feature in several Archaea. Although many unusual pathways have been unravelled in different Archaea, the knowledge about their regulation is rather limited.

T. tenax is a facultatively heterotrophic hyperthermophile and therefore represents an ideal organism to study the carbon flux in response to autotrophic and heterotrophic growth conditions ("carbon switch").

Within the present study, the DNA microarray technology (focussed approach; 105 different CCM genes) has been established for *T. tenax* in order to analyse the mode and significance of transcriptional regulation of the CCM. First studies were performed, to monitor gene expression changes upon the switch from heterotrophic (in the presence of glucose) to autotrophic conditions (in the presence of CO_2).

Transcriptional profiling revealed a highly coordinated gene expression of the reversible Embden-Meyerhof-Parnas (EMP) pathway and the citric acid cycle (CAC) for controlling the catabolic and anabolic carbon flux, whereas the branched Entner-Doudoroff (ED) pathway, exhibited no strong regulation on gene level.

The catabolic flux (heterotrophic growth) is enforced by the enhanced expression of the three EMP genes *pfp*, *fba* and *gor* encoding PP_i-dependent phosphofructokinase, fructose-bisphosphate aldolase and ferredoxin-dependent GAP oxidoreductase as well as the CAC genes *acn*, *idhA*, *gltA*-2, *sdhA-B-C-D* coding for aconitase, isocitrate dehydrogenase and for the key enzymes citrate synthase 2 and succinate dehydrogenase.

The autotrophic flux is driven by induction of the EMP genes *gap, pgk* and *pps* encoding classical GAP dehydrogenase, phosphoglycerate kinase and phosphoenolpyruvate synthetase as well as the CAC genes *oorA-B-C-D* and *frdA-B* coding for the reversible 2-oxoglutarate-ferredoxin oxidoreductase and fumarate reductase.

This study in combination with available biochemical data (Brunner *et al.*, 1998, 2001; Schramm *et al.*, 2000; Tjaden *et al.*, 2006) spot key regulation points of the *T. tenax* EMP variant at the level of GAP and PEP/pyruvate conversion. At both regulation sites three different genes/enzymes are responsible for the control of the carbon flux (GAPDH (*gap*), GAPN (*gapN*), GAPOR (*gor*) and PK (*pyk*), PPS (*pps*), PPDK (*ppdk*), respectively).

From comparable studies of two other hyperthermophilic, heterotrophic Archaea, *P. furiosus* and *S. solfataricus* as well as of the halophilic *H. volcanii* it can be concluded that GAP conversion seems to represents a conserved key regulation point in Archaea, whereas regulation at PEP/pyruvate conversion seems to be less conserved. Interestingly, another conserved regulation site might be situated at the upper part of the EMP pathway (fructose 6-phosphate/fructose 1,6-bisphosphate conversion), which is exclusively executed on gene level.

Since whole genome sequence information of *T. tenax* is available and the DNA microarray technology is established for *T. tenax*, the construction of a whole genome DNA microarray is planned in the near future. The experiments will allow a global transcriptional profiling of the *T. tenax* genome providing valuable insights into the regulatory network linking the CCM with other metabolic pathways, e.g. amino acid metabolism.

To get more insights into the molecular background of the regulation of CCM in *T. tenax* the functional genome organisation of CCM genes was analysed in order to identify transcriptional regulators. The gene coding for a Lrp homolog (leucine-responsive regulatory protein, bacterial-type global transcription regulator) was identified downstream of the *gad* gene belonging to the ED gene cluster of *T. tenax* and the properties of its gene product have been analysed. DNA binding studies with the recombinant protein demonstrated that Lrp binds to its own promoter region and to the promoter region of the ED gene cluster, thus suggesting an involvement in transcriptional regulation of the ED genes. However, additional work is required in order to confirm this first studies and to identify growth conditions, which induce differential ED gene expression.

In addition to the regulation of the CCM in dependence of the carbon source, it is also a matter of great interest how *T. tenax* adapts to environmental stress, e.g. high temperature and osmolarity or oxidative stress. Therefore, the metabolism of the compatible solute trehalose was further investigated in the course of this study.

Initial studies revealed that trehalose is synthesised via the OtsA/OtsB pathway in *T. tenax* (Brenner, 2001; Zaparty, 2003, Siebers *et al.* 2004). The *tpsp* and the *gt* gene are part of the trehalose operon of *T. tenax*. The clustering of the *tpsp* and *gt* gene with an additional ORF coding for a putative mechanosensitive channel (*msc*; MscTTX) in the trehalose operon of *T. tenax* (*msc-gt-tpsp*), suggests a functional relation of all three gene products.

Functional analysis of the recombinant proteins shows that the pathway is characterised by the first reported bifunctional trehalose-6-phosphate synthase/phosphatase (TPSP), which is

activated by the putative glycosyl transferase (GT; TPSP activating protein). However, the mode of activation is still unclear.

The results of the present study lead to a proposed model of stress response in *T. tenax* that comprehends regulation of cell turgor, e.g. under osmotic stress. In future studies this model will be proven by detailed functional analyses of TPSP activation by GT and of the recombinant MscTTX.

The current work supports a role of trehalose as compatible solute rather than as carbon and energy source in *T. tenax*.

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LIST OF ABBREVIATIONS

AA	acetaldehyde
A. bidest.	aqua bidestillata = two times distilled water
aa	amino acid
Amp^r	ampicillin resistence
APS	ammonium persulfate
bp	base pair(s)
β-Me	beta-mercaptoethanol
1,3BPG	1,3 Bisphosphoglycerate
BSA	bovine serum albumin
CAC	citric acid cycle
CAM^r	chloramphenicol resistence
CAPS	3-[cyclohexylamino]-1-propanesulphonic acid
CDP	disodium 2-chloro-5 (4-methoxyspiro{1.2-dioxetane-3.2'-(5'-chloro) tricycle [3.3.1.1.$^{3.7}$] decan}-4-yl)-1-phenylphosphate
CE	crude extract
CI(A)P	calf intestinal (Alkaline) phosphatase
CoA	coenzyme A
Conc.	concentration
Cy3-dUTP	5-amino-propargyl-2'-deoxy-uridine-5'-triphosphate with Cy3-fluorochrome (fluorescence at 535 nm)
Cy5-dUTP	5-amino-propargyl-2'-deoxy-uridine-5'-triphosphate with Cy5-fluorochrome (fluorescence 635 nm)
DEPC	diethylpyrocarbonate
DIG-UTP	digoxigenin-labeled UTP
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dsDNA	double-stranded DNA
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH = German Collection of Microorganisms and Cell Cultures
DTT	dithiothreitol (Clelands reagent)
e.g.	for example
ED	Entner-Doudoroff pathway
EDTA	ethylene-diamine-tetraacetic acid
EMP	Embden-Meyerhof-Parnas pathway
Ery4P	erythrose 4-phosphate
et al.	et alteri = and others
$F1,6P_2$	fructose 1,6-bisphosphate
F6P	fructose 6-phosphate
FAD^+	flavin adenine dinucleotide (oxidized)

$FADH_2$	flavin adenine dinucleotide (reduced)
Fd_{red} /Fd_{ox}	reduced / oxidized ferredoxin
Fig.	figure
g	gram
x *g*	gravitational acceleration
G1P	glucose 1-phosphate
G6P	glucose 6-phosphate
GA	glyceraldehyde
GAP	glyceraldehyde 3-phosphate
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
(6x) His-tag	(hexa) histidine tag
HP	heat precipitation
i.e.	id est = that is, that is to say
IPTG	isopropyl-ß-D-thiogalactopyranoside
Kan^r	kanamycin resistence
kb	kilobases
kDa	kilodalton
KDG	2-keto-3-deoxygluconate
KDPG	2-keto-3-deoxyphosphogluconate
l	liter
LB	Luria-Bertani
LrP	Leucine-responsive regulatory protein
M	molar (mol/l)
m	milli (10^{-3})
µ	micro (10^{-6})
µCi	micro curie
mA	milliampere
min	minute
MOPS	3-(N-morpholino)propanesulphonic acid
MsCS	mechanosensitive channel (of small conductance)
MW	molecular weight
n	nano (10^{-9})
$NAD(P)^+$	nicotinamide adenine dinucleotide (phosphate) (oxidized)
NAD(P)H	nicotinamide adenine dinucleotide (phosphate) (reduced)
NCBI	National Center for Biotechnology Information
Ni-NTA	nickel-nitrilotriacetic acid
OA	oxalacetate
OD	optical density

ORF, *orf*	open reading frame
Ots	osmoregulatory trehalose synthesis
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PG	phosphoglycerate
pH	negative logarithm of the hydrogen ion (H^+) concentration
Pi	inorganic phosphate
PMSF	phenylmethylsulphonyl fluoride
PPi	inorganic pyrophosphate
Psi	pound-force per square inch
Pfu-Polymerase	DNA-polymerase from *Pyrococcus furiosus*
R5P	ribose 5-phosphate
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
rNTP	ribonucleoside triphosphate
rpm	rounds per minute
rpoS	stationary-phase sigma factor encoding gene of *E. coli*
RT	room temperature
Ru5P	ribulose 5-phosphate
s.	see
SDS	sodiumdodecylsulfate
sec	seconds
sp.	species
SSC	standard saline citrate
ssDNA	single-stranded DNA
Tab.	table
TAE	tris-acetate-EDTA buffer
Taq-Polymerase	DNA-Polymerase from *Thermus aquaticus*
TBE	tris-Borate-EDTA buffer
TBP	TATA binding protein
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFB	transcription initiation factor B
TLC	Thin layer chromatography
T_m	melting temperature
TPS	trehalose-6-phosphate synthase (domain)
Tre	trehalose
Tre6P	trehalose 6-phosphate
Tris	tris-(hydroxymethyl)-aminomethane

U	(enzyme) Unit
U/ADPG	U/ADP glucose
UV	ultraviolet
V	volt
Vol	volume
W	watt
www	world wide web
x	fold
Xyl5P	xylulose 5-phosphate
<	lower than
>	higher than
% (v/v)	percent by volume
% (w/v)	percent by weight

Amino acids (1- and 3-letter code)

A	alanine(ala)	I	isoleucine (ile)	R	arginine (arg)
C	cysteine (cys)	K	lysine (lys)	S	serine (ser)
D	aspartic acid (asp)	L	leucine (leu)	T	threonine (thr)
E	glutamic acid (glu)	M	methionine (met)	V	valine (val)
F	phenylalanine (phe)	N	asparagine (asn)	W	tryptophan (trp)
G	glycine (gly)	P	praline (pro)	Y	tyrosine (tyr)
H	histidine (his)	Q	glutamine (gln)		

Nucleosides and nucleotides

A	adenosine	AM/D/TP	adenosine mono/di/triphosphate
C	cytidine	CTP	cytidine triphosphate
G	guanosine	GTP	guanosine triphosphate
T	thymidine	TTP	thymidine triphosphate
U	uridine	UD/TP	uridine di/triphosphate

APPENDIX

Tab. A1 **Probe generation.** ORF ID, primer sets, annealing temperatures, probe size, quantitation of probes.

No.	ORF	size (bp)	primer set and sequence (5'-3')		probe size (bp)	T_m	PCR	conc. [ng/µl]
1	TTX_0059	303	TtxA-1f	CTTGTGTGAACGAGGAGGAGGC	256	64°C	63°C	250
			TtxA-1rev	TGGAGCGAGTCCGAGATCTGCC		65.8°C		
2	TTX_0060	906	TtxA-2f	TGGTGAGCAGAGATGGGCGAGT	840	64°C	60°C	200
			TtxA-2rev	ACTTCTTCAGAGTATCCGGCGGC		64.2°C		
3	TTX_1762, N-terminus	442	TtxA-3f	ATGAAAACCACTATAAGCATAATCAAG	434	57.4C	60°C	410
			TtxA-3rev	TTATACATAGGCAAGTTGAACGCGC		61.3°C		
4	TTX_1762, C-terminus	693	TtxA-4f	ACAGCCGGCCTTGTGATAGACC	633	64°C	56°C	350
			TtxA-4rev	TGTTCGCCAACCATGTATACTCCA		61°C		
5	TTX_0980	897	TtxA-5f	ATGAGATAGGGCCAGACGATAGAG	729	62.7°C	60°C	385
			TtxA-5rev	CTAACCCCACGTGCTTCAAGAAAG		62.7°C		
6	TTX_1277	1014	TtxA-6f	CATAGCAGTCTACACGTTCGTGAA	943	61°C	60°C	162
			TtxA-6rev	GGTCTCGTACAGCCTCATCCAAT		62.4°C		
7	TTX_1278	792	TtxA-7f	CTTAAGGATATTCGCGAGGAGGG	740	62.4°C	60°C	237
			TtxA-7rev	TTCCGCCGTACACCAACTCTGC		64°C		
8	TTX_0494	687	TtxA-8f	GCCGAGAGAGCTGCCAGAGAG	528	65.7°C	62°C	375
			TtxA-8rev	GCTTGCCAACAGGACCCCCCT		65.7°C		
9	TTX_1518	1035	TtxA-9f	TTGAAGTTTTTGAAGTACCTAGATATG	977	57.4°C	56°C	200
			TtxA-9rev	CAAGCCCGACTCGCCCAATATG		64°C		
10	TTX_1169	1506	TtxA-10f	CGTCCCCGTCTACCCCTCCTA	505	58.5°C	58°C	275
			TtxA-10rev	AGCCGATGCCCTCTCTGAATACT		58.5°C		
11	TTX_1534	1032	TtxA-11f	AGGATACGGGACTATAGGAAAGCG	990	62.7°C	60°C	187
			TtxA-11rev	GTCACAATGCCCAACGATTTATCAG		61.3°C		
12	TTX_2037	1878	TtxA-12f	ATGCGCGTCGCCTTTATAGATCTC	461	62.4°C	60°C	262
			TtxA-12rev	TCATACCTATCGAACTTCCAATCCT		65.8°C		
13	TTX_1535	1206	TtxA-13f	TATATATAAATGTGCTGAACCTAATTCG	768	57.8°C	56°C	212
			TtxA-13rev	AGGCCCGCCCGCCTGTTGATA		64°C		
14	TTX_2061	552	TtxA-14f	ATAGAGAGCATACACGACAAATATAAA	515	57.4°C	55°C	425
			TtxA-14rev	TCTGAGAGACCCAAGTAGCTAGC		62.4°C		

15	TTX_1889	1239	TtxA-15f	ATGGACACAACTATCGAGGAGCTC	709	62.4°C	60°C	100
			TtxA-15rev	AGATGAAATTCTGCCCCACTTGCC		65.7°C		
16	TTX_1891	1341	TtxA-16f	TGGGGCCTTCGACTGATAGACTG	770	64.2°C	62°C	125
			TtxA-16rev	GTAATTTAATAGTATGTGTGCCGCC		64°C		
17	TTX_0910	2433	TtxA-17f	GTCTCCAGACTCGTCCGCGTG	676	65.7°C	63°C	225
			TtxA-17rev	CCCTCCTCCTCAAGCAACCTGT		67.6°C		
18	TTX_0683	2748	TtxA-18f	ATGCCTAAAAAGTACGTCTTCGATTT	726	58.5°C	56°C	375
			TtxA-18rev	GGATCTATGAGCCTTACTACTACG		61°C		
19	TTX_0329	1044	TtxA-19f	AGTCGTTGAGGCTGAGGGAGGT	986	64°C	60°C	212
			TtxA-19rev	CTTTATATCGTCGTGGGTCCACAC		62.7°C		
20	TTX_1156	1494	TtxA-20f	AGCGTCCGTCAGCTCCATCTTC	719	64°C	62°C	350
			TtxA-20rev	GTTCCTCTAACGACCCACACCG		64°C		
21	TTX_1156a	861	TtxA-21f	GAGCCATCCCTTAATACCTTTTCG	882	61°C	58°C	400
			TtxA-21rev	GCCCTCAGCCAAGCCTTCTCC		65.7°C		
22	TTX_1157	933	TtxA-22f	CCCCTCATACAGCTCAACGCAG	851	64°C	62°C	112
			TtxA-22rev	TCCCCTCTGACTCCGACCACC		65.7°C		
23	TTX_1396,	789	TtxA-23f	CGATTACAAGGCCGCCCGCGA	676	65.7°C	62°C	187
	C-terminus		TtxA-23rev	CCTCTTCAAAGTGCTCCTCCTCG		64.2°C		
24	TTX_1396,	1067	TtxA-24f	AGAAGGAAGTTCGTCGTACAAGAG	996	61°C	58°C	275
	N-terminus		TtxA-24rev	AAGCCGAGAGTGTTGCCGAGAGAT		64.4°C		
25	TTX_1294	1818	TtxA-25f	AGTGCGCCTCACCGTTTTAGTTG	702	64°C	62°C	175
			TtxA-25rev	GCGTTCGTCCCACCCTCTTAGT		67.6°C		
26	TTX_1768,	899	TtxA-26f	GGCTATATTCAGGGTTGCGAGGA	889	62.4°C	60°C	775
	N-terminus		TtxA-26rev	GATTGGGCACATCATACAGGCGT		62.4°C		
27	TTX_1768,	675	TtxA-27f	TGGCTGAGGGCGACTACAGACTG	633	66°C	62°C	150
	C-terminus		TtxA-27rev	GGAACTCCTCCAGACCCAACCT		64°C		
28	TTX_1787	1587	TtxA-28f	GCAAAGTTACCTACGTCTCTGTGT	711	64.2°C	62°C	187
			TtxA-28rev	TAATATACCCCATTTACGCCTCTGC		65.7°C		
29	TTX_1101	1434	TtxA-29f	TCAAGGGAGGAGGTGAGGAGGG	648	65.7°C	62°C	1488
			TtxA-29rev	CTCAGTGAACAACAACATAGTGCTT		65.8°C		
30	TTX_0788	1197	TtxA-30f	CTATCGGCAATAGACCTAAAAAGGG	483	62.4°C	61°C	1175
			TtxA-30rev	GCTCTAACAGCGGCTTCATTCGA		64°C		
31	TTX_0789	1704	TtxA-31f	GGCCATAGGATTCTCAGACGAAG	721	62.4°C	60°C	250

			TtxA-31rev	TAAGAGCCCGCCGACATAGTTAG		64°C		
32	TTX_2039	681	TtxA-32f	CGTAAAGCAGCGGAGTATATAAGG	590	61°C	59°C	275
			TtxA-32rev	TCTATGACGCCAGGTATCCCCC		64°C		
33	TTX_1754	735	TtxA-33f	GCATCCACATAGGCTCCTCCCT	727	64°C	62°C	225
			TtxA-33rev	TAGCGACATGTCACCCTCTTGGC		64.2°C		
34	TTX_1753	496	TtxA-34f	ATGTCGCTAGTGGAGGAGCTTAC	440	62.4°C	60°C	225
			TtxA-34rev	TAGGTCTCACACGAGTCGGCGG		65.8°C		
35	TTX_0613	672	TtxA-35f	TGATAGATCACACAATACTTAACCCC	625	60.1°C	58°C	225
			TtxA-35rev	GTCTTGATAATGTCCACTCCGTGG		62.7°C		
36	TTX_1882	1317	TtxA-36f	GTCTTCCTCCCTATATCTATCCAG	583	67.6°C	62°C	487
			TtxA-36rev	TCACTTTGATCGACGCAGCGGC		65.7°C		
37	TTX_0497	1125	TtxA-37f	TTAGAGGGAATAATAGTAAAAGAAAGTA	732	62.7°C	60°C	237
			TtxA-37rev	AGGGATATACCTAAGGCCGACTG		67.6°C		
38	TTX_1513	1233	TtxA-38f	AGGACGACCGGCAAGATCATACA	495	62.7°C	58°C	350
			TtxA-38rev	TTCTCCCAATATTCTAATACATGCGC		61.3°C		
39	TTX_1436	1116	TtxA-39f	GTCATAAAAATCGAGCCACCCTCA	495	61°C	58°C	637
			TtxA-39rev	TCATGCCCTTATTACCCCCTTCTC		62.4°C		
40	TTX_1435	1005	TtxA-40f	TCGCCTCAAGCGATCGCAAGATA	950	62.4°C	60°C	150
			TtxA-40rev	CGAGCTGGTAGTGGACGTAGTC		64°C		
41	TTX_1437	1146	TtxA-41f	TCAGACCAAGGCCGGATCAGCG	701	64.2°C	60°C	150
			TtxA-41rev	CCACTTCTCTAGGATATGCATATGC		62.7°C		
42	TTX_0493	2640	TtxA-42f	TATAAGATCTACAAGCTGAGGGCTC	549	64°C	63°C	100
			TtxA-42rev	ACGGCGATGGCTGTATATAGGTG		65.7°C		
43	TTX_1489	1320	TtxA-43f	ATGTCAGATTACCTAGAGAAAATAAAG	687	62.7°C	60°C	137
			TtxA-43rev	TTTACAACTTTCCTTTCCTCATCGG		64°C		
44	TTX_0334	1449	TtxA-44f	CCCAAACTGGATAAATACGACGTC	690	67.6°C	64°C	387
			TtxA-44rev	GCCACCGCAAGGGCTATCTCG		67.6°C		
45	TTX_0406	1401	TtxA-45f	GTGGCTATGATCAACGACGGACC	655	62.4°C	60°C	188
			TtxA-45rev	GGAGAAGGAGGGGAAGGCTGG		64°C		
46	TTX_2034	1191	TtxA-46f	CGTCGCTGCTCTCCGTCAAGAG	770	65.8°C	62°C	337
			TtxA-46rev	TATCCTCAACCCCATATATATCGTC		64°C		
47	TTX_2033	948	TtxA-47f	CGGCCCAGCTATAGCAATGCGT	823	64°C	60°C	312
			TtxA-47rev	AGCTCTCTCCAGCTCCTCTATCT		62.4°C		

48	TTX_2036	522	TtxA-48f	GATCGAGATAAGGTTCCACGGAAG	520	62.7°C	58°C	200
			TtxA-48rev	TCATAGTTCCCTAGTCTGGTCGTA		61°C		
49	TTX_2035	309	TtxA-49f	ACCGATAGGCGGCATAATCACTG	252	62.4°C	60°C	312
			TtxA-49rev	ACGGGACACTCGTGGGCGCAT		65.7°C		
50	TTX_0209	1197	TtxA-50f	GAGGATTGCTCTGACTGGAAACC	724	62.4°C	60°C	200
			TtxA-50rev	CTCCCCCTTCGCCACTCTCTC		62.4°C		
51	TTX_0210	1023	TtxA-51f	TTCAAAATAAACGTCAGAGGGCTGC	999	61.3°C	60°C	125
			TtxA-51rev	GTCCACTGGGCCGATGAATTCTC		64.2°C		
52	TTX_0208	936	TtxA-52f	GTGGATAGGGAGAGGGGGACAG	886	65.8°C	62°C	162
			TtxA-52rev	GGGGCACACGTCGGCACATATG		65.8°C		
53	TTX_0922	612	TtxA-53f	GTCCTCGTAGGCCCTGATACTAA	520	62.4°C	60°C	375
			TtxA-53rev	TATTGATCCCCAACCCCGCGCG		65.8°C		
54	TTX_0921	1164	TtxA-54f	TCGCAAAATACGGAGTCAAAATACC	654	59.7°C	54°C	125
			TtxA-54rev	TCCTCGTTGGTCCCCTTCATCC		62.4°C		
55	TTX_0864	1749	TtxA-55f	TGATCATAGGCTCAGGTATTGCGG	771	62.7x°C	60°C	125
			TtxA-55rev	CTTCCTTTCCTCCAACTTAGTCCA		64°C		
56	TTX_0863	714	TtxA-56f	GGCGAGAGGAGTTGGTGGCAG	637	65.7°C	60°C	237
			TtxA-56rev	GTTGTATAGCGAAGCCGGGATCA		62.4°C		
57	TTX_0862	483	TtxA-57f	ATGAGCGATCACAACAGTAGGCG	468	62.4°C	60°C	112
			TtxA-57rev	GCCTCCGAATGCCACAAGATATG		62.4°C		
58	TTX_0861	351	TtxA-58f	ATGAGCGAGGGATTAATCAGGCTG	351	62.7°C	56°C	100
			TtxA-58rev	CTACAAGGTCATTATAGCATAGATAAC		58.9°C		
59	TTX_1104	1746	TtxA-59f	CCGGATCTCGAGGTGTGCGTAG	653	61°C	60°C	162
			TtxA-59rev	TATAGGCGATAGTGTGGACCAGC		67.6°C		
60	TTX_1105	357	TtxA-60f	ATTGACGGTGAAACGATATAGAGAC	346	59.7°C	58°C	737
			TtxA-60rev	CTAAAGTGGGAGCCCGTGAGGA		64°C		
61	TTX_1106	1092	TtxA-61f	GTTATAGAGGAGGTAGACGTAGAG	979	61°C	58°C	262
			TtxA-61rev	TATAGAAGTCGCGGGCCATAAGC		62.4°C		
62	TTX_1295	943	TtxA-62f	TTGAAGAGAGGGCGAGGAGGGT	928	64°C	62°C	512
			TtxA-62rev	TGGGCCGCGGCCAACATAACG		65.7°C		
63	TTX_1765	870	TtxA-63f	GCCAGCATATCGCCCGCCTTC	765	65.7°C	60°C	175
			TtxA-63rev	GGCCCAACAACTGAACATAACGG		62.4°C		
64	TTX_1764	609	TtxA-64f	GGACTCCACTTTCCGACGACGA	586	64°C	62°C	362

			TtxA-64rev	CTGAACAGCTCGCCGACCTCTC		65.8°C		
65	TTX_1427	927	TtxA-65f	TTGATAGATATAATAAAAGGGCTCCC	772	58.5°C	56°C	175
			TtxA-65rev	TCTCCTCAGGCGACAACTCGAC		64°C		
66	TTX_1514	1305	TtxA-66f	ATTCCTAAGGTTCCAATTACATCAATG	674	58.9°C	56°C	487
			TtxA-66rev	CTCAACGGCTTGCGCGCAAGC		58.8°C		
67	TTX_1316,	705	TtxA-67f	TGTAGTAGAGGAGTTGATAGAGCG	625	61°C	60°C	200
	N-terminus		TtxA-67rev	TGTGATTATGGCGGGGGCGGG		65.7°C		
68	TTX_1316,	1743	TtxA-68f	CGGCTCTGGCGTCTACTTCTAC	755	61°C	60°C	900
	C-terminus		TtxA-68rev	GCCTCTCAACTTCCTCCCTCGG		62.4°C		
69	TTX_2058	1311	TtxA-69f	CATCAGAGGGATATATAACAAGGAC	574	59.7°C	56°C	287
			TtxA-69rev	GGAAAGGCCCAGCGTACCCAAA		65.7°C		
70	TTX_0995	897	TtxA-70f	ATGAGATAGGGCCAGACGATAGAG	520	64°C	61°C	162
			TtxA-70rev	CTAACCCCACGTGCTTCAAGAAAG		62.4°C		
71	TTX_1336	684	TtxA-71f	GTGGAGAAGCCTAGGGAGCCG	533	65.7°C	62°C	1212
			TtxA-71rev	CTTACGACGCTACGCCTACCCA		64°C		
72	TTX_1335	969	TtxA-72f	TCGGGGGGGCTGGGTTTATGG	898	65.7°C	62°C	112
			TtxA-72rev	AGGGGGCGCCACCACCACTCG		68.8°C		
73	TTX_0596	978	TtxA-73f	CTGGCGCACATGATAAGAGACAC	887	62.4°C	60°C	300
			TtxA-73rev	GTCCTCGACTACCTCCCTATTTG		62.4°C		
74	TTX_1400	876	TtxA-74f	GCAAGTCCTGCGTCCTCTACAAC	748	64.2°C	60°C	275
			TtxA-74rev	CTCGCTCTCTCGTAACAATCTTTG		61°C		
75	TTX_1397	1833	TtxA-75f	GGCCCGGCGGCAAATATGAGTT	533	64.2°C	60°C	250
			TtxA-75rev	GTATTCCTTAAACAGCCTCTCCATG		62.4°C		
76	TTX_1399	1482	TtxA-76f	AACGAACTTCGACTTTTATAACTGGA	650	61.3°C	59°C	587
			TtxA-76rev	CCTCAGCTGAGGCCTCGTCCA		64°C		
77	TTX_1158	1821	TtxA-77f	GGACAAAAACTTCTACGTGGCGG	775	65.7°C	62°C	150
			TtxA-77rev	GGACTGACACTGGGCCTCTATC		65.7°C		
78	TTX_1745	2127	TtxA-78f	TGGCAAGAGGACCGTGAGGATAC	730	64.2°C	62°C	137
			TtxA-78rev	AGCTCCACTACGTCTCCCGCG		64°C		
79	TTX_1304	2208	TtxA-79f	TCCAACAGATTGCCCGTCACTATA	711	64°C	62°C	300
			TtxA-79rev	GCGCACCTCCACCACCTTCTTG		64°C		
80	TTX_1304a	796	TtxA-80f	ATGTACCAGCCCACCGCCACC	744	65.7°C	62°C	150
			TtxA-80rev	CCCCACGCGATAGGCATAAGCA		64°C		

81	TTX_1305	1164	TtxA-81f	GTGAGGGCGCTCAACAGATATGC	710	67.6°C	60°C	175
			TtxA-81rev	CCCCCATCTCGGCCAACACATC		61°C		
82	TTX_0218	651	TtxA-82f	TCTTCAAGTCGGCGTATTATCTATC	614	59.°C	58°C	412
			TtxA-82rev	TCATAACAAGGCCCTCTCCAGCG		64.2°C		
83	TTX_0217	1206	TtxA-83f	GAACGCCATATTTAAATACGCCGAA	709	64.2°C	62°C	100
			TtxA-83rev	AATAGAGGATCGTCATGAGGTAGC		64.2°C		
84	TTX_0328	855	TtxA-84f	TGGCCGCACGGTAGTATGATACC	850	64.2C	62°C	187
			TtxA-84rev	ATAGCCCCAGCGCCCTCCTCA		65.7°C		
85	TTX_0327	441	TtxA-85f	ATGAGGGTCAGAGTCAAAGTCAAC	411	61°C	60°C	400
			TtxA-85rev	CTGATAGCCGGTGCATCTACACA		62.4°C		
86	TTX_0326	2042	TtxA-86f	CGACGACATAGAGCTCCCCGG	778	65.8°C	62°C	275
			TtxA-86rev	GGATGTGGTAGAAGGCGAAGTTG		64°C		
87	TTX_1758	1914	TtxA-87f	TTTTTAATAGGAGGGCCGCAGGG	743	62.4°C	60°C	250
			TtxA-872rev	TCGAGCACTTCGTCCACTGTAATG		62.7°C		
88	TTX_1757	948	TtxA-88f	TAAGATAACGCTTAAGAGGACGCC	848	61°C	60°C	125
			TtxA-88rev	TCAGATAGTTGGGGTCGTACATGG		62.7°C		
89	TTX_1455,	462	TtxA-89f	GGTTCATGGAAATAACTGTCAGAATA	446	58.5°C	56°C	150
	N-terminus		TtxA-89rev	TCATAGCGCGTCCGAGAGAACGC		66°C		
90	TTX_1455,	1161	TtxA-90f	GAGAAAGTAGCTCCCGCCAAAGT	729	62.4°C	60°C	262
	C-terminus		TtxA-90rev	TCGCCCCACCACTTGAGGATGC		65.8°C		
91	TTX_1454	909	TtxA-91f	CTACAGATCGACAAAAAGCCCATG	822	61°C	60°C	250
			TtxA-91rev	TCCTCCCAAGAGTTTAACCGCCT		62.4°C		
92	TTX_1785	1152	TtxA-92f	GCGCTCACGGCCCTCAAGACT	710	64°C	62°C	125
			TtxA-92rev	CTATGCCCGCCACGACGTCCA		65.7°C		
93	TTX_1786	924	TtxA-93f	CGTCAGAATAGACCAGCTCCCG	908	64°C	62°C	150
			TtxA-93rev	CGGCCCCATCCACTCTCCTCT		65.7°C		
94	TTX_0712	1953	TtxA-94f	GAACCTCATCTCAATCTGCCCAC	723	62.4°C	60°C	200
			TtxA-94rev	CGTTACACAACGAAGGATCTATCC		61°C		
95	TTX_0713	627	TtxA-95f	CTCTATGTGCGCCCAAGTCTGTC	567	64.2°C	63°C	287
			TtxA-95rev	GCCCCCATCTAGCCAGTCCAC		65.7°C		
96	TTX_0388	537	TtxA-96f	GCTTCCTCCGGGCAGAAAACCC	511	65.8°C	60°C	200
			TtxA-96rev	AGACCTCTTGAACCTTTCTACTGC		61°C		
97	TTX_1550,	495	TtxA-97f	ATGGCGATTATACCAATATTAGGGTT	474	58.5°C	56°C	262

	N-terminus		TtxA-97rev	CAGTGATTCTGCTGAAAATAAGATAGA		58.9°C		
98	TTX_1550 ,	1644	TtxA-98f	TATATACACTAAGGCCGCAGACCT	673	64°C	62°C	162
	C-terminus		TtxA-98rev	GCCCGAGAGCGCTATACCCATG		64°C		
99	TTX_0826	897	TtxA-99f	CGTTGGTGACGCATAGACATGCT	788	62.4C	60°C	200
			TtxA-99rev	CGGATCTCCCTCCCTCAGCAC		65.7°C		
100	TTX_1664	978	TtxA-100f	TTAAGCGGGCGTGAAAATTTATTGAC	933	60.1°C	58°C	250
			TtxA-100rev	GGCCTTGATCGTCATACTTTCTTTT		59.7°C		
101	TTX_1913,	342	TtxA-101f	GTGTCAGCCTCCTATCTATCGCC	301	64.2°C	61°C	312
	C-terminus		TtxA-101rev	GCCATATCGTCTGCACGCCATTC		64.2°C		
102	TTX_0682	618	TtxA-102f	TCAGATCGGCGTGGAAAAGACTC	517	62.4°C	60°C	237
			TtxA-102rev	CTTATTACAACATCGGCGGCTTCT		61°C		
103	TTX_0683	936	TtxA-103f	TGACGTAACCGTAAAATTAACACAAC	814	58.5°C	56°C	250
			TtxA-103rev	GTCGCCCGAGTAGATCCCAATC		64°C		
104	TTX_0482	1401	TtxA-104f	TAATAAAATATACTCCATGAAAACTTCTG	771	56.8°C	55°C	125
			TtxA-104rev	GACCATCCCGGCGAGCTTCTG		61°C		
105	TTX_1140	1368	TtxA-105f	CGGCTGTCGCCGCATATCTTGC	732	61°C	60°C	137
			TtxA-105rev	GTTTACTATCGCCACGCCCATTAT		65.7°C		
106	TTX_0155	2661	TtxA-106f	GTATATCTCGCCCACGCCCAAAC	707	62.7°C	60°C	237
			TtxA-106rev	TGTTCAACATACAGCCTACGCCG		61°C		
107	TTX_0083	1635	TtxA-107f	CCTCAACAATAATAGCGGTCGTGG	727	62.7°C	60°C	112
			TtxA-107rev	GACTATCTTAATGCCTGGCTTGGC		64.2°C		
108	TTX_2085	903	TtxA-108f	CAGGATCTAATATGCCCGGTGTG	842	62.4°C	58°C	125
			TtxA-108rev	TTGCCCAACTCATTTAAAAGCTCTC		59.7°C		
109	TTX_1484	999	TtxA-109f	GCTATTTAAGCTTAATCACAGAGACA	910	58.5°C	56°C	273
			TtxA-109rev	AGCGCCTTGGCCAACTCCTTGT		64°C		
110	TTX_0178	612	TtxA-110f	ACTCTTCTAGAGCCGCCGGCC	573	65.7°C	63°C	556
			TtxA-110rev	CTCCGCCCTCCTCGACGTACA		65.7°C		
111	TTX_0985	342	TtxA-111f	AAGGACTTATCACAATTTCAGAGGG	314	59.7°C	58°C	400
			TtxA-111rev	GGATCCACTTGTATGCCGTATTTG		61°C		

Tab. A2.1 **Results of the single hybridisation experiments (1-7)**

	Experiment No.																	
	1		2		3		4		5		6		7					
sequences	Ri	Log2	Ri	Log2	Ri	Log2	Ri	Log2	Ri	Log2	Ri	Log2	Ri	Log2	N spots	average	SD	*p* value
1			1.406772498	0.492389036	1.002159496	0.003112135	1.813075759	0.858439209							11	0.45131346	0.429140423	0.1359
2	0.796658598	-0.327966494			0.629658159	-0.667359292	2.331992408	1.221563092	2.302473958	1.203184839	1.135622711	0.183483606	2.354939374	1.235689919	27	0.474765945	0.860346463	0.0061
3	0.862585483	-0.213260659			0.631941694	-0.662136641	2.385283757	1.254160902	2.351019112	1.233286267	1.026848271	0.038223022	2.014204921	1.010210467	32	0.443413893	0.827056289	0.0413
4	0.720697795	-0.472533663	3.213202456	1.684011884	0.570473319	-0.809768681	2.578336381	1.366440497	2.440921087	1.287425655	1.102342159	0.140572095	2.721096834	1.444188298	36	0.662905155	1.022160416	0.0028
5	0.974592006	-0.037129705	0.933346588	-0.099515185	0.825227044	-0.277136994	2.457467066	1.297172082	2.706364964	1.436356406					25	0.463949321	0.830303721	0.0318
6			0.059704611	-4.066013833	0.189664096	-2.398481499	0.16013412	-2.64264736							14	-3.035714231	0.900578811	0.0008
7	0.161088859	-2.634071374	0.075402198	-3.729249609	0.144311995	-2.79273687			0.080077869	-3.642452611					15	-3.199627616	0.566276544	0.0000
8	0.767065773	-0.382577806	0.828614896	-0.271226339	0.720490899	-0.472947888	0.609302574	-0.71476926							16	-0.460380323	0.188594924	0.0413
9	0.588111808	-0.765837639			0.492184297	-1.022729464	0.900927958	-0.150516349	1.478884147	0.564509039					17	-0.343643603	0.707447234	0.0159
10	1.035770702	0.050704656	0.666286381	-0.585785691	0.644691411	-0.633319331	1.134388008	0.181914186							19	-0.246621545	0.42293153	0.1900
11							4.262523302	2.091707721	6.758197763	2.756638568	3.232638889	1.692712356	3.875731991	1.954468811	18	2.123881864	0.453146095	0.0000
12	0.265351301	-1.914024471	0.265670802	-1.912288417	0.240586966	-2.055369607									11	-1.960560832	0.082111396	0.0000
13	3.147889387	1.654384847	3.717524947	1.894324424			5.935648376	2.56940563	8.065444709	3.011754082	7.159940209	2.83994754	5.902090209	2.561225971	28	2.421843416	0.535024805	0.0000
14	1.056047733	0.078675046	0.989442457	-0.015312288	0.872498621	-0.196775244	1.077988816	0.10834221	1.291062127	0.368558426	0.651026674	-0.619211439			32	-0.045953882	0.335699367	0.0391
15	1.038773052	0.054880493	1.404882636	0.490449613			1.477339601	0.563001501	1.41874421	0.504614505	1.131001879	0.177601326	0.804984345	-0.312967369	35	0.246263345	0.340815544	0.0041
16	1.014730847	0.02109711			1.081550289	0.113100748	0.836176878	-0.258119945	0.570619672	-0.80939861	0.571897436	-0.806171658			29	-0.347898471	0.441512994	0.2271
17			11.92742644	3.576210884			8.091325732	3.016376102	21.60842144	4.433521778	11.1028009	3.472851766	22.76233647	4.508576748	32	3.801507456	0.647030416	0.0000
18	0.800658906	-0.320740334			0.480769231	-1.056583528									12	-0.688661931	0.520319712	0.0085
19	0.64399053	-0.634888622	0.776362022	-0.365198549	0.564324747	-0.825402478			1.34003473	0.422270392	1.064902831	0.090721795	1.124177563	0.168869926	31	-0.19060459	0.492914174	0.0424
20	0.984264507	-0.022882023			0.758511868	-0.398756342	0.769861415	-0.377329329	0.61078049	-0.711274116	0.351036009	-1.510309067			24	-0.604110175	0.562162444	0.0002
21	0.670646729	-0.576375086			0.773089331	-0.371292967	1.217435132	0.283844903	1.169546627	0.22594938	0.530840611	-0.913649348			16	-0.270304624	0.517474623	0.1194
22	0.611590821	-0.70936134			0.874760374	-0.193040226	1.189268947	0.250075011	1.278471708	0.354420234	0.755651511	-0.404207044			25	-0.140422673	0.445365005	0.2160
23															NF			
24	0.672643266	-0.572086516	0.884305828	-0.177382697			0.841512284	-0.248943763	1.087528974	0.121053838	0.582708507	-0.779153722			27	-0.331302572	0.351300506	0.0084
25															NF			
26	0.159715802	-2.646421039	0.383221938	-1.383747944	0.284069197	-1.815685694	4.236021359	2.082709864	2.613588241	1.386031869	1.168848698	0.225088191			32	-0.358670792	1.884281201	0.0258
27	0.13388645	-2.900918136	0.451694473	-1.146580835	0.28079315	-1.832420354	3.802628945	1.92699717	2.477464275	1.308864254	0.904001635	-0.145602713	5.820529686	2.541150449	37	-0.035501452	2.040588355	0.4835
28	0.603386781	-0.728845004	0.637824779	-0.648767948			1.261914858	0.335614574			0.588248288	-0.765502878			24	-0.451875314	0.527251202	0.0106
29															NF			
30			2.247411358	1.168264214	1.397182235	0.482520204	3.423360192	1.775413095							13	1.142065838	0.646844474	0.0001
31			2.066413712	1.047129122			3.492504316	1.804261899	2.239652427	1.163274857	1.870383897	0.903334415			21	1.229500073	0.397651496	0.0000
32	1.412126669	0.497869506	1.542213162	0.625002186			1.720921298	0.783181121							11	0.635350938	0.142937056	0.0066
33	0.882882031	-0.179707414	1.644824747	0.717933876	0.580114078	-0.785591464	2.469390094	1.30415476	2.380075962	1.251007619					18	0.461559475	0.917269277	0.0101
34	0.698279425	-0.518123632	1.540881477	0.623755896	0.57793429	-0.791022624	2.568894198	1.361147473					1.664384646	0.734988884	19	0.2821492	0.90528042	0.1820
35	1.256976437	0.329957606			0.888936045	-0.169848468	0.954248664	-0.067562834							12	0.030848768	0.264036286	0.7803
36	2.037546424	1.02683293	0.787696581	-0.344288081	1.422769041	0.508701488	1.09313822	0.128475831							11	0.329930542	0.581029759	0.3947
37	0.433032481	-1.207452853	1.492166262	0.577408294	0.186606822	-2.421926362	7.179832098	2.843950107			2.103393844	1.072719009			35	0.172939639	2.046112639	0.0013
38			0.365867773	-1.450605752	0.460433854	-1.118934182					0.457970071	-1.126674776			20	-1.23207157	0.189295723	0.0006
39					0.276293188	-1.855728099	1.592076118	0.670909313	1.131558398	0.178311041	0.540342142	-0.88805489			30	-0.473640659	1.12796875	0.0004
40	0.438288328	-1.190047837			0.370960989	-1.430660617	1.343920657	0.426447966	1.024331089	0.034682105	0.445504289	-1.166488775			30	-0.665213432	0.835795412	0.0000
41	0.601278627	-0.733894418					1.826570026	0.869137064	1.264536104	0.338608228	0.602540079	-0.730870887			30	-0.064255003	0.801314174	0.1148
42	0.567103225	-0.818316735					0.416504815	-1.26359492	0.447063106	-1.161449604	0.247768832	-2.012933378	0.426491925	-1.22940967	30	-1.297140861	0.43753755	0.0000
43					1.059847283	0.083856397	0.440922246	-1.181403826	0.259720791	-1.944966586			0.167609063	-2.576827938	23	-1.404835488	1.144760985	0.0003
44															NF			
45															NF			
46	0.713597143	-0.486818256			0.554746702	-0.850098909	0.836342333	-0.257834506	0.481934118	-1.053092157	0.348350566	-1.521388187	0.591118163	-0.758481545	36	-0.821285593	0.442266437	0.0000
47	0.633381152	-0.658854158			0.60502418	-0.724935293	0.764612658	-0.387199012	0.406031826	-1.300335279	0.490795353	-1.026806506	0.460960404	-1.117285266	34	-0.869235919	0.337534106	0.0001
48	0.725437109	-0.463077548			0.537421715	-0.895873479	0.450807158	-1.149417673					0.302683865	-1.724116324	21	-1.058121256	0.52671492	0.0018
49	0.645385593	-0.631766722			0.596676595	-0.744978907	0.674065539	-0.569039224	0.452071181	-1.145378145	0.290889133	-1.781458694	0.322263083	-1.633689164	24	-1.084385143	0.524787201	0.0235
50					3.015468377	1.592382106	3.52437277	1.817366525			5.074124279	2.343158855	9.262397856	3.211385728	24	2.241073303	0.719318049	0.0000
51					2.846828219	1.509355442	4.139918412	2.049602336			5.731375748	2.518881482	9.485861478	3.245778801	24	2.330904515	0.736297968	0.0000
52					2.755314492	1.462216998	3.175254822	1.666872377			3.756723529	1.909474947	4.685309365	2.22814431	16	1.816677158	0.329646338	0.0000
53	0.714899176	-0.484188306	1.16025469	0.214441529			1.459944974	0.545913994	1.288247816	0.365410147					19	0.160394341	0.450579172	0.6505
54	0.541882622	-0.883947713	0.980345466	-0.028637861			1.387122051	0.472094734	1.133377097	0.180627955	0.625475396	-0.676974959	0.745067594	-0.42455678	30	-0.226899104	0.522939369	0.2430
55							0.212038197	-2.237603919	0.197978762	-2.336582418	0.087981579	-3.506654699	0.088050296	-3.505528329	19	-2.896592341	0.704948219	0.0000

56							0.346378062	-1.529580536	0.295015375	-1.761137952	0.112104143	-3.157088503	0.181136661	-2.46484953	18	-2.22816413	0.735992065	0.0000
57			0.454682593	-1.137068322			0.542086684	-0.883404527	0.393912239	-1.344053853	0.190612054	-2.391288738	0.236288898	-2.081376248	20	-1.567438338	0.641459837	0.0000
58			0.414908367	-1.269135344			0.500964641	-0.997219315	0.356292167	-1.488867328	0.156560823	-2.675204852	0.246605237	-2.019724656	17	-1.690030299	0.66649708	0.0002
59							8.405360188	3.071309643			9.034111666	3.175382746	25.41254125	4.667468747	9	3.638053712	0.893016958	0.0232
60			4.942846072	2.305341979			2.611827694	1.385059723			5.410353535	2.435722869			12	2.042041524	0.572685436	0.0006
61			3.310697798	1.727135327			5.181298515	2.373313705	50.92352055	5.670260257	14.1163311	3.819293269			22	3.397500639	1.749516548	0.0000
62			0.646783102	-0.628646107	0.649956567	-0.621584782	0.568528043	-0.814696581	0.728694305	-0.45661438					19	-0.630385462	0.146341444	0.0000
63	0.837868621	-0.25520405	0.497769294	-1.006450858	0.720524368	-0.472880872	0.856811394	-0.22295043	0.724866228	-0.464213321			0.9341398	-0.09828962	34	-0.419998192	0.321919126	0.0033
64															NF			
65	0.663412881	-0.59202107	0.723178834	-0.467575641	0.484728551	-1.044751033			0.66995906	-0.577855156	0.494074924	-1.01719826	0.610345889	-0.712301031	34	-0.735283699	0.241970901	0.0001
66			0.822994869	-0.281044659	2.192478723	1.132562842	0.669494598	-0.578855681	0.733835991	-0.446470432					13	-0.043451982	0.793419212	0.6903
67															NF			
68															NF			
69	0.548643371	-0.866059421	0.469762385	-1.089996897	0.476124668	-1.070588719	1.233877988	0.303199741	1.137993711	0.186492585	0.830997103	-0.267084647			33	-0.46733956	0.62799977	0.0027
70	0.729852601	-0.454322963	1.217911712	0.284409554			1.162338304	0.217030032			0.822161836	-0.28250569			19	-0.058847267	0.365311578	0.4110
71															NF			
72															NF			
73	0.686589314	-0.542480691	0.738219494	-0.43787826	0.794557637	-0.331776218	1.242879182	0.313686062	1.208574249	0.273306108	0.679452291	-0.557555841			30	-0.21378314	0.401457951	0.1136
74	1.173284016	0.230552288	1.136926932	0.185139538											11	0.207845913	0.032111664	0.4684
75	0.667393141	-0.583391236	1.166457919	0.222134261			0.996040344	-0.005723916			0.702062822	-0.510327963			16	-0.219327213	0.390614324	0.9946
76	1.375820313	0.460292061	1.113893835	0.155611737	0.991963987	-0.01164035	1.322391064	0.403148881							12	0.251853082	0.219869971	0.2076
77	1.161257374	0.215687758			0.889735265	-0.16855196	1.415440669	0.501251278	1.468242285	0.554090057					12	0.275619283	0.331328462	0.0512
78	0.741232576	-0.432001807			0.731213582	-0.451635226	1.280137547	0.356298832	1.575678054	0.655972791	0.937310721	-0.093400711			21	0.007046776	0.488959228	0.7107
79															NF			
80	1.369949479	0.45412269	0.997363824	-0.003808219	1.789448252	0.839514824	1.212593494	0.278095986							16	0.39198132	0.352971678	0.1581
81															NF			
82															NF			
83	1.83614208	0.876677699	0.689391909	-0.536603729	0.809890979	-0.304200378	2.165374668	1.114616672							10	0.287622566	0.828755811	0.4737
84	0.743302537	-0.427978562	0.779709561	-0.358991269	0.558699622	-0.83985525	1.301706252	0.380403921	1.783607162	0.834797898	0.662276258	-0.594494955	0.753070476	-0.409143209	25	-0.202180204	0.59071658	0.0390
85	0.80284861	-0.316800125	0.979750074	-0.029514318			1.445636835	0.531705172	1.256005971	0.328843323	0.870065485	-0.200804106	0.972814699	-0.039763068	36	0.045611147	0.322955992	0.4489
86	0.744011778	-0.426602635	0.927559271	-0.108488622			1.282301917	0.358735984	1.211879634	0.277246415	0.90240059	-0.148160084	1.157880269	0.211486078	35	0.027369523	0.303809117	0.8318
87			1.333301373	0.415002917	1.679446862	0.747986149	0.328746027	-1.604954638	0.49791903	-1.006016939	0.289170089	-1.790009762	0.204280105	-2.291379392	36	-0.921561944	1.239238164	0.0378
88	2.394789776	1.259899016	1.490659722	0.575950966	2.014726548	1.01058404	0.490950869	-1.026349437	0.654210183	-0.612173879			0.514892188	-0.957657713	36	0.041708832	1.027148062	0.4025
89															NF			
90															NF			
91															NF			
92	0.332911523	-1.586789289			0.269236542	-1.893053862			0.413529218	-1.273938828	0.346017057	-1.531084936			22	-1.571216729	0.254169468	0.0000
93	0.528599559	-0.919752872	0.182589044	-2.453327896	0.416710571	-1.262882395	0.712670496	-0.488692896	0.491782522	-1.023907633	0.45599473	-1.132910944			36	-1.213579106	0.662372157	0.0000
94	3.675987177	1.878131734	0.540782729	-0.886879018	2.01176962	1.008465103	0.123393587	-3.018660675	0.195867362	-2.352051076	0.215289568	-2.21564968	0.219161533	-2.189933495	32	-1.110939587	1.872653931	0.2056
95	4.733411312	2.242880291	0.550545299	-0.86106682	6.996717937	2.806678333	0.106753816	-3.227640459			0.075678091	-3.723980488	0.142671762	-2.809228277	29	-0.928726237	2.851373491	0.1617
96			3.048363013	1.608034716							2.668071768	1.415797474	3.79523074	1.924187601	14	1.64933993	0.25669967	0.0001
97			0.396681984	-1.333945218	0.80135315	-0.319489928	0.812443892	-0.299659911	0.81704193	-0.291517976	0.300131205	-1.73633477			20	-0.796189561	0.689480203	0.0182
98	1.101194277	0.139069017	0.459473913	-1.121945143	0.835007676	-0.260138636	0.883142932	-0.179281145	0.846223925	-0.240888619	0.305618831	-1.710194657	0.355292252	-1.492921869	34	-0.695185865	0.731042638	0.0001
99															NF			
100															NF			
101															NF			
102															NF			
103															NF			
104	0.466437123	-1.10024548	0.336964743	-1.569330447	0.466164485	-1.101088999	4.36039336	2.124458289	1.904478537	0.929396029					19	-0.143362121	1.593743366	0.7243
105							4.896667999	2.291800383	3.480619455	1.799344089	2.012161415	1.008746042	3.299203915	1.72211795	17	1.705502116	0.528613622	0.0000
106			1.962258299	0.972514961			4.936272676	2.30342209	4.69199215	2.2302006	1.996552923	0.997511314	4.655657217	2.21898484	26	1.744526761	0.694151971	0.0001
107	1.18365654	0.243250517					11.12141619	3.475268606	5.349594734	2.419429602	2.465277778	1.301750213			20	1.859924735	1.396134645	0.0002
108	1.587387492	0.666654343	1.127635763	0.173301139							1.694815397	0.76112814	4.182663164	2.06442182	9	0.916376361	0.807595662	0.0161
109	0.718821136	-0.476295264	0.380893544	-1.392540259	0.315612648	-1.663773072	0.838776908	-0.253640952	1.113074808	0.154550558			0.891441457	-0.165788039	27	-0.632914505	0.727514543	0.0040
110	1.9708178	0.978794407	1.941275066	0.957004553			2.594209444	1.37529496	3.056235892	1.6117559	1.846672317	0.88492789	3.002996457	1.586402773	32	1.232363414	0.331826201	0.0000
111	2.513946001	1.329953662			2.007455817	1.005368235	1.997025168	0.997852515	2.219130882	1.149994758	1.395980047	0.481278321	1.46932003	0.555148661	32	0.919932692	0.334634585	0.0016

Tab. A2.2 **Data of the control experiment: Comparison of two independent autotrophically grown *T. tenax* cultures**

sequence	F635 Median - B635	average	SD	F532 Median - B532	average	SD	Rm	Log2
2	3517			4045				
2	5719			5306				
2	4862			5010				
2	3838			4762				
2	3489			4729				
2	3779	4201	897.3179295	4291	4690	461.1521966	0.895580196	-0.1591
3	4314			4383				
3	4520			4296				
3	5512			4353				
3	6739			5545				
3	5745	5101.5	1092.82327	5873	4789.891667	719.9843036	1.065055403	0.0909
4	9998			13549				
4	10586			12195				
4	9075			8783				
4	9952			10044				
4	10798			12939				
4	10358	10127.83333	611.2689806	13953	11910.48333	2061.875867	0.850329332	-0.2339
5	1082			1216				
5	1403			1652				
5	1434			1724				
5	1052			734				
5	1159			1166				
5	1515	1274.166667	199.8473584	1632	1353.908333	385.1614096	0.94110261	-0.0876
9	1233			785				
9	2007			1536				
9	1667			1601				
9	1383			1201				
9	1380	1534	307.6263968	1080	1240	336.1626957	1.236608115	0.3064
10	1423			1346				
10	1143			1264				
10	1067			1467				
10	1181			1637				
10	1699			1981				
10	1020	1255.5	258.436646	711	1401.083333	422.6525034	0.89609231	-0.1583
11	3204			3518				
11	2212			1678				
11	2922			2499				
11	3080	2854.5	443.6075593	3269	2741.0375	830.9828191	1.041393998	0.0585
13	1184			1436				
13	1174			1049				
13	1160			876				
13	1156			643				
13	1251	1185	105.0252351	749	950.47	310.4917483	1.246751607	0.3182
14	885			1371				
14	609	747	126.7990536	597	983.875	547.548136	0.75924279	-0.3974
15	2295			1550				
15	2316			1700				
15	3140			4273				
15	3101			3619				
15	2919			3085				
15	3372	2857.166667	451.0474107	2701	2821.291667	1067.942573	1.012715807	0.0182
16	905			1252				
16	589	747	148.6865831	485	868.7	542.1387691	0.859905606	-0.2177
17	6202			4852				
17	4890			3317				
17	5979			4423				
17	11087			13295				
17	9244			9850				
17	9985	7897.833333	2527.609417	9771	7584.408333	3954.720323	1.041324911	0.0584

19	2335			2146				
19	2045			1614				
19	1805			1227				
19	2002			1369				
19	3129			3952				
19	2565	2313.5	480.9265017	3089	2232.808333	1080.338486	1.036139092	0.0512
20	641	641	158.1123019	656	656.2		0.97683633	-0.0338
21	937	937	129.5077218	1218	1218.05		0.769262346	-0.3785
22	930			1512				
22	887			1403				
22	965			1261				
22	1047			804				
22	931	952	66.61055972	1432	1282.48	282.3455122	0.742311771	-0.4299
24	2006			2066				
24	1917			3036				
24	1734			2589				
24	1743			1995				
24	1428			1036				
24	1269	1682.833333	283.4455268	864	1931.2	850.3242981	0.871392571	-0.1986
26	11016			12158				
26	8943			9285				
26	13190			14213				
26	11593			14192				
26	10481			12983				
26	8354	10596.16667	1770.392207	10111	12156.84167	2073.151946	0.871621673	-0.1982
27	23400			24485				
27	23010			22927				
27	18631			18513				
27	24246			20163				
27	26186			23712				
27	25011	23414	2606.41969	22970	22128.19167	2295.231403	1.058107249	0.0815
28	1874			2426				
28	2489			2744				
28	2137			2308				
28	1605			1751				
28	1869			2088				
28	2042	2002.666667	299.5574514	2478	2299.108333	343.6899786	0.871062332	-0.1992
29	5980			5609				
29	3638			3857				
29	3685			3700				
29	2907			3021				
29	4119			3909				
29	5056	4230.833333	1109.496357	4855	4158.625	921.6271745	1.017363512	0.0248
30	1182			1429				
30	1084			1547				
30	1010			1357				
30	1139			1437				
30	1223			1238				
30	888	1087.666667	123.0945436	1141	1357.875	147.3908333	0.801006475	-0.3201
31	4121			3858				
31	4912			5047				
31	5071			5146				
31	4232			4547				
31	3969			4160				
31	5000	4550.833333	495.521308	4476	4539	497.5911906	1.002607035	0.0038
32	841			1017				
32	659	750	71.97777435	857	936.7	112.9956636	0.80068325	-0.3207
33	2010			2333				
33	3168			3094				
33	3246			3406				
33	2766			2893				
33	2275			2285				
33	1282	2457.833333	753.2697835	1498	2584.85	687.5855903	0.950861107	-0.0727
34	2748			3198				
34	2984			3976				

34	2785			4140				
34	2707			4243				
34	3213	2887.4	210.8371409	4363	3984.12	461.9620582	0.724727167	-0.4645
35	1726			1856				
35	1387			1536				
35	1884			1689				
35	1868			2116				
35	2131			2206				
35	2082	1846.333333	269.7885592	2110	1918.591667	268.6776813	0.962337826	-0.0554
36	1026	1026		1309	1309		0.783804431	-0.3514
37	1308			813				
37	1716			1132				
37	2121			2258				
37	2039			1777				
37	1862			1352				
37	2016	1843.666667	299.4466007	1459	1465.116667	505.2583801	1.258375328	0.3316
38	8476			7973				
38	8144			6072				
38	10081			11641				
38	7329			6715				
38	4564			3230				
38	3405	6999.833333	2527.69598	2126	6292.833333	3417.446016	1.112350028	0.1536
39	1147			1251				
39	1097			917				
39	1079	1107.666667	125.6702033	671	946.3333333	291.3731728	1.170482564	0.2271
40	1465			1321				
40	1270			876				
40	1246			826				
40	1317			1471				
40	1081	1276	138.1690993	932	1085	291.1416893	1.175549167	0.2333
41	1071			576				
41	1331			1590				
41	1444	1282	232.3201814	1356	1173.85	530.5273485	1.092132726	0.1271
42	1354			1665				
42	1097			1016				
42	1067	1173	157.7540279	1305	1329	325.3535308	0.882666566	-0.1801
46	3436			3539				
46	2791			4312				
46	3181			3862				
46	2765			2402				
46	2255			1727				
46	2217	2774.166667	486.5948691	1689	2921.733333	1132.553048	0.949493451	-0.0748
47	2073			1428				
47	1873			1267				
47	2706			3154				
47	2467			2362				
47	2443			1765				
47	2436	2333	302.9831678	1750	1954.291667	697.7371234	1.193782914	0.2555
48	1798			2166				
48	2033			1814				
48	1859			2827				
48	1700			2134				
48	1381			1133				
48	1169	1656.666667	321.9407813	785	1809.65	745.0586259	0.915462474	-0.1274
49	1849			2152				
49	1800			2506				
49	2281			1887				
49	2236			2287				
49	2508			2723				
49	2227	2150.166667	272.6722697	2428	2330.416667	291.357716	0.922653317	-0.1161
50	13224			17277				
50	11928			15833				
50	13091			14516				
50	11591			14901				
50	12589			17729				

50	8961	11897.33333	1573.538772	12205	15410.21667	2018.586376	0.772041925	-0.3732
51	10833			12946				
51	9945			10404				
51	7092			8446				
51	11125			10841				
51	11518			11809				
51	11444	10326.16667	1683.458038	12674	11186.85	1669.608159	0.923062941	-0.1155
52	3991			6855				
52	4460			6726				
52	3979			5510				
52	5729			6867				
52	4765			6790				
52	5325	4708.166667	711.8163855	7812	6759.908333	733.6888465	0.696483803	-0.5218
53	1772			1841				
53	1626			1825				
53	1499			1792				
53	1306			1546				
53	1784			1587				
53	1686	1612.166667	182.8993348	1609	1700	133.1698014	0.948333333	-0.0765
54	4281			4599				
54	3562			5059				
54	4513			5755				
54	4640			5506				
54	3146			3137				
54	2572	3785.666667	829.0285078	3422	4579.8	1085.307348	0.82660087	-0.2747
55	1601			1561				
55	2038			2108				
55	2148			2593				
55	2304			2515				
55	1742			2423				
55	2160	1998.833333	270.9689404	2110	2218.075	381.1488525	0.901156784	-0.1501
56	1704			1979				
56	2290			2230				
56	1659			2514				
56	2197			2740				
56	2372			2609				
56	1543	1960.833333	364.6463584	1359	2238.333333	510.8636243	0.876023827	-0.1910
57	4865			6009				
57	4120			3981				
57	6017			5745				
57	6520			7574				
57	6810			6429				
57	5098	5571.666667	1045.198673	6234	5995.191667	1171.324478	0.929355887	-0.1057
58	3343			4644				
58	2435			3106				
58	2491			3389				
58	2781			4320				
58	3422	2894.4	465.3609352	4644	4020.33	724.7847375	0.7199409	-0.4740
59	1147			1223				
59	1032			1228				
59	1037			899				
59	1257			1295				
59	1561			1495				
59	1694	1288	278.8347181	1515	1275.991667	224.6583393	1.009410981	0.0135
60	5788			8428				
60	6237			8511				
60	4242			5713				
60	4461			6306				
60	5096			8029				
60	6032	5309.333333	838.6078146	8514	7583.416667	1246.411396	0.700124174	-0.5143
61	3419			2488				
61	4382			3260				
61	4905			5793				
61	4195			4695				
61	4609			3922				

61	5291	4466.833333	643.5055296	4170	4054.641667	1144.457805	1.10165921	0.1397
63	1135			763				
63	1010			582				
63	1587			1063				
63	2085			2545				
63	1604			1897				
63	2054	1579.166667	447.9390211	1748	1433.241667	756.3367474	1.101814651	0.1399
65	3574			3269				
65	2950			2144				
65	2739			1895				
65	2967			2223				
65	3452			4210				
65	3291	3162.166667	326.3074726	3836	2929.383333	976.2029511	1.079464961	0.1103
66	911	911		1131	1131		0.805232687	-0.3125
67	1064			1017				
67	1118	1091	129.5021235	1356	1187	239.2142241	0.919433676	-0.1212
69	1867			1912				
69	1224			1142				
69	1247			831				
69	1110			592				
69	1661	1422	356.0423664	1105	1117	497.2509444	1.273375367	0.3487
70	1308			1244				
70	1502			2191				
70	931	1247	290.3463449	1488	1641	491.7742809	0.759871628	-0.3962
73	2110			2013				
73	2845			2603				
73	1635			1186				
73	1898			1507				
73	2184			1947				
73	2953	2270.833333	523.7775927	2512	1961.233333	552.2783453	1.15785985	0.2115
76	990			1475				
76	999			1440				
76	964			1217				
76	1178			1134				
76	1139			1233				
76	948	1036.333333	97.14456581	1298	1299.366667	133.5010624	0.797568046	-0.3263
77	1248			1319				
77	1356			1502				
77	1317			1468				
77	1322			1673				
77	1349			1252				
77	1685	1379.5	154.4781538	1595	1468.233333	160.0716485	0.939564556	-0.0899
78	3782			4230				
78	3288			4541				
78	3973			5346				
78	5060			5641				
78	2611			2667				
78	2883	3599.5	882.4290906	3256	4279.891667	1158.252857	0.841025961	-0.2498
80	904			1070				
80	870			1023				
80	924			1278				
80	846			1193				
80	863	881.4	31.80880381	1092	1131.18	102.8787976	0.779186336	-0.3600
83	1385			1578				
83	1443			1500				
83	1903			1810				
83	2076			2085				
83	2298			2410				
83	2017	1853.666667	364.4917923	2048	1905.133333	342.662682	0.972985268	-0.0395
84	3187			4764				
84	3059			3749				
84	3498			4480				
84	2800			3976				
84	3316			5117				
84	3322	3197	243.7785881	4659	4457.4	510.7437743	0.717234262	-0.4795

85	1514			950				
85	2038			1403				
85	4412			5327				
85	3218			3402				
85	2662			2547				
85	3237	2846.833333	1020.251031	2373	2666.875	1566.010967	1.067479103	0.0942
86	3246			3486				
86	2914			2809				
86	3459			4882				
86	2940			3551				
86	2418			2190				
86	1761	2789.666667	614.9141946	1282	3033.225	1242.492163	0.919703176	-0.1208
87	1094			674				
87	1100			595				
87	1903			2270				
87	1533			1557				
87	1578	1441.6	371.8157698	1448	1308.83	692.1981667	1.101441746	0.1394
88	2675			3101				
88	2429			2101				
88	1864			1612				
88	2425			3219				
88	1849			2137				
88	1690	2155.333333	403.2164018	1372	2257.033333	758.2731168	0.954940852	-0.0665
92	5852			8564				
92	4350			6763				
92	4837			5700				
92	4685			4304				
92	4314			3751				
92	5414	4909	610.9237814	7015	6016	1800.489869	0.815933023	-0.2935
93	1931			1352				
93	3415			4236				
93	3013			3259				
93	2759			2391				
93	2426			1747				
93	3329	2812	565.5554497	2601	2598	1043.096425	1.08254285	0.1144
96	2515			2874				
96	1799			2039				
96	3100			4635				
96	1973			2705				
96	1660			1659				
96	1255	2050.333333	659.0568008	888	2466.7	1284.563467	0.831204984	-0.2667
97	3788			4894				
97	2989			3592				
97	4171			3512				
97	3975			4366				
97	4417			5928				
97	3940	3880	487.4464073	5333	4604.308333	963.398027	0.842689003	-0.2469
98	6909			9078				
98	6960			7433				
98	7078			8132				
98	5492			7499				
98	4349			6584				
98	4579	5894.5	1252.700243	7542	7711.341667	832.6525406	0.764393572	-0.3876
100	2043			2329				
100	1407			1789				
100	2028			2001				
100	1473			1607				
100	1249			1493				
100	1153	1559	386.1742698	1624	1807	310.5706995	0.862548359	-0.2133
103	1127			1085				
103	1042	1085	60.1040764	861	973.25	158.6747617	1.114307732	0.1561
104	1036			1144				
104	1976			2311				
104	1697			2107				
104	1111			1535				

104	1064			1250				
104	1955	1473	452.725708	2254	1766.866667	521.2970839	0.833773535	-0.2623
105	6089			7441				
105	13733			12462				
105	6149			6658				
105	9668			13131				
105	7974			8948				
105	7117	8455	2907.433576	8293	9488.691667	2684.535094	0.891060675	-0.1664
106	21510			18334				
106	17572			20107				
106	18074			22801				
106	14815			18187				
106	14322			18973				
106	16011	17051	2635.503266	16291	19115.36667	2192.672275	0.891987424	-0.1649
107	13661			16223				
107	15635			14890				
107	14139			11638				
107	17802			17600				
107	21593			24460				
107	14703	16256	2996.225876	16402	16868.95833	4244.450311	0.963633894	-0.0534
108	3276			3001				
108	2192			2305				
108	2680			2600				
108	1992			2343				
108	1855	2399	581.4903267	2580	2565.64	277.5198583	0.935049344	-0.0969
109	1416			818				
109	1597			1004				
109	2312			2801				
109	2631			2412				
109	2272			1644				
109	1849	2013	467.924531	1220	1649.708333	800.0889609	1.220114667	0.2870
110	5723			6679				
110	5031			5534				
110	5122			6942				
110	4251			5818				
110	3309			4712				
110	3490	4488	965.8409117	4276	5660.15	1052.577484	0.792852957	-0.3349
111	2075			1594				
111	1209			722				
111	1374			813				
111	4209			5008				
111	4192			3832				
111	2886	2658	1334.082868	2242	2368.525	1723.70081	1.122006312	0.1661

Tab. A2.3 **Complete data set of Cy5 (Median of F635(-background)) and Cy3 (F532(-background)) intensities**

Slide	1		2			3			4			5			6			7		
sequence	F635 Median - B635	F532 Median - B532*	sequence	F635 Median - B635	F532 Median - B532*	sequence	F635 Median - B635	F532 Median - B532*	sequence	F635 Median - B635	F532 Median - B532*	sequence	F635 Median - B635	F532 Median - B532*	sequence	F635 Median - B635	F532 Median - B532*	sequence	F635 Median - B635	F532 Median - B532*
2	2013	1540	2	1804	602	2	7040	9095	1	5017	3766	1	1961	2028	1	1983	1110	2	1387	508
2	1820	1510	2	1616	678	2	6185	7890	1	4553	3370	1	1771	1809	1	1858	1020	2	2121	860
2	2644	2297	2	2449	1032	2	3956	4947	1	5067	3446	1	1391	1369	1	1360	739	2	2870	1305
2	2670	2473	2	1586	692	2	6143	7551	1	4153	2776	1	1671	1574	2	14858	6024	2	3684	1698
2	3254	3101	2	1416	667	2	4016	4836	4	20771	6893	2	10251	16636	2	22285	9837	3	2248	854
3	867	677	2	1611	781	3	7732	9325	4	19002	5982	2	13945	21791	2	22512	10131	3	3117	1248
3	1536	1478	3	1746	773	3	6811	8182	4	24291	7560	3	9522	16395	2	26731	11683	3	3816	1803
3	1264	1219	3	1727	851	3	6710	7815	4	25834	7971	3	7958	13136	2	25696	10744	4	1180	373
3	1690	1644	3	1706	857	3	5992	6780	4	22878	7057	3	7763	11848	2	20889	8600	4	2261	891
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4	3122	2496	3	1259	672	4	4647	6617	5	6271	7824	4	16912	30272	3	19745	8252	5	801	177
4	3228	2744	4	2018	573	4	4642	6572	5	4124	5049	4	24414	42169	3	20743	8541	5	1302	416
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4	3441	3072	4	1752	654	4	5833	8192	5	5941	5768	5	4533	5569	3	14191	5844	7	116	1449
4	4525	4242	4	1814	700	4	3931	5236	5	5635	5457	5	2320	2740	4	20533	7998	9	742	457
4	4766	5009	4	2455	980	4	3251	4275	6	2240	41111	5	2622	2871	4	17915	6629	9	843	615
11	2278	667	4	3309	1439	5	1892	2141	6	2317	41447	6	2976	16327	4	27431	10987	11	1325	30
11	2214	689	11	953	196	5	1915	2085	6	2755	47634	6	1832	9023	4	26051	10150	11	2813	465
11	2025	660	11	1319	352	5	1155	1220	6	3159	53953	7	873	6523	4	26411	10383	11	1154	288
13	1363	166	11	1024	302	5	1620	1680	6	2764	43761	7	735	5340	4	23831	8993	13	2691	205
13	1199	149	13	1073	182	5	1238	1127	6	3391	50565	7	842	5617	5	3939	1673	13	2198	218
13	1514	200	15	1360	1447	5	1079	877	7	921	15264	7	748	4680	5	3860	1657	13	3061	409
13	1848	262	15	1475	1736	7	1065	7163	7	1555	22196	8	3553	4931	5	2933	1167	13	2557	471
13	1631	235	15	855	1017	7	1019	6356	7	1777	22990	9	2063	4418	5	5889	2400	14	553	343
13	2025	326	15	839	1063	7	926	5652	7	1857	23995	9	1635	3400	5	4783	1870	14	761	486
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16	1033	1849	26	4370	714	11	1313	523	12	495	1909	16	9987	9376	9	3071	3485	16	1004	1900
16	757	1374	27	10005	1654	11	1740	674	12	941	3333	16	12882	12067	9	2326	2558	17	9029	323
16	541	1434	27	11839	1968	12	394	1634	12	745	2612	16	8626	7923	9	2426	2636	17	9546	343
17	6028	511	27	9232	1578	12	298	1199	13	5060	1426	16	10771	9826	10	4594	4035	17	11406	454
17	10129	889	27	9888	1697	12	387	1449	13	5939	1646	16	14899	13247	10	5477	4833	17	10307	422

17	9755	857	27	8036	1427	12	388	1452	13	3983	1103	18	4011	8812	10	4214	3831	17	9574	528
17	8947	816	27	8253	1512	12	336	1060	13	4852	1337	18	4778	10127	10	5463	4970	17	10662	730
17	8563	792	34	1414	717	13	1143	561	13	4622	1205	18	5021	10368	10	5796	4901	19	888	589
17	7793	748	34	1005	736	13	1179	356	13	6151	1515	18	7204	14764	10	7164	6263	19	1118	757
19	1970	1429	38	2096	1793	13	1002	269	14	4520	4907	18	4250	8664	11	7366	1787	19	1361	1008
19	1784	1678	38	2416	2068	13	1075	283	14	2811	2940	18	3954	8038	11	13401	3107	19	1035	810
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19	1432	1537	38	2542	2211	14	1209	1480	14	4885	4832	19	9997	17573	11	10414	2490	19	730	607
19	1306	1418	38	2687	2411	14	1036	897	14	3712	3647	19	8714	15300	11	8730	2033	20	507	783
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20	461	1309	42	476	1114	15	4238	4139	15	11584	7820	20	1347	1689	13	8543	1528	22	664	469
20	268	1360	42	589	1387	15	5137	4854	15	18006	12070	21	7088	9236	13	6590	1081	22	670	493
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21	538	1152	43	179	979	15	4500	3929	17	34135	3623	21	5352	6933	14	3610	3182	22	736	577
22	1173	1297	43	201	1119	16	5040	5401	17	34534	3306	21	3971	5041	14	1866	1902	22	872	720
22	915	1211	43	305	1781	16	3198	3210	17	22977	1828	22	2185	2615	14	2616	2554	22	719	618
22	748	996	43	177	1265	16	3311	3234	17	28258	2092	22	2258	2644	14	2694	2597	24	1535	1300
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24	1758	2303	46	832	1252	16	3868	3548	17	25674	1780	22	1165	1205	15	9401	6655	24	1472	1324
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24	997	2075	47	556	1026	17	1592	201	19	6759	8619	25	1080	1410	15	12868	8317	26	2828	900
24	888	1986	47	470	929	18	2263	3332	19	5841	7251	26	3801	16277	16	3816	4637	26	3447	1252
26	3818	3053	47	601	1315	18	1936	2481	19	5647	6664	26	14040	46528	16	4604	5615	26	6747	2661
26	2988	2514	47	663	1488	18	1767	2112	23	3751	4894	27	12895	48765	16	3028	3483	26	5135	2134
26	5899	5065	47	691	1563	18	1688	1994	23	3071	3692	27	14422	54053	16	2667	3144	27	3959	1483
26	3416	2990	47	670	1599	18	1648	1946	23	3437	4006	27	7693	27834	16	3612	4226	27	7855	3044
26	4663	4159	48	524	1460	18	1544	1682	23	3103	3574	27	14526	45762	16	3516	4301	27	9771	3962
27	5297	5212	48	632	2034	19	4308	7418	23	3434	3862	30	10389	7436	17	28366	3596	27	10068	4156
27	7736	8651	48	502	1697	19	3433	5523	23	2243	2486	31	2425	2233	17	32598	3979	27	5455	2333
27	4898	5617	48	1024	3669	19	2741	4272	24	6577	7957	31	1766	1618	17	42298	5188	31	1689	701
27	5952	6940	49	374	1026	19	3458	5178	24	7464	8606	31	2376	2129	17	22035	2792	31	2270	1067
28	621	862	49	374	1139	19	2689	3887	24	6491	7458	31	1846	1459	17	25691	3071	33	569	239
28	1030	1591	49	320	1051	19	2984	4177	24	7062	7940	33	6561	13819	17	22994	2876	37	4533	885
28	840	1322	49	356	1202	20	1841	2117	24	9098	9971	33	716	1234	19	4208	3283	37	3969	804
28	844	1465	50	3038	270	20	1426	1585	24	10977	11975	34	3095	5512	19	5127	4079	37	3674	750
28	1202	2131	50	5055	455	20	1137	1071	25	1025	1979	34	2928	4910	19	5734	4490	37	4229	942
28	789	1682	50	2451	282	20	1343	1216	25	1992	3306	35	3854	4336	19	5900	4677	37	3233	750
31	2325	1030	50	4736	545	20	1361	1232	25	1172	1750	36	4254	3051	19	4111	3223	37	3725	1101
31	1438	769	50	2979	367	21	2011	3433	25	2251	3330	36	2955	2016	19	6790	5022	39	1067	792
31	1756	974	50	3164	395	21	1922	3201	26	10247	28728	37	4158	23029	20	1294	1674	39	1220	1016
31	2139	1194	51	3549	351	21	1384	2061	26	13762	36790	37	4801	26356	20	1555	1964	39	1236	1061
31	1560	961	51	2899	303	21	1124	1489	26	16389	43659	37	5515	29931	20	1904	2496	39	1277	1115
37	2142	988	51	2520	264	21	1586	2079	26	14189	36190	37	4145	22024	20	2468	3379	39	1045	1015
37	1961	906	51	2403	258	21	1303	1649	26	15731	39594	37	5252	27872	20	1401	1735	39	1067	1109
37	2276	1069	51	2410	262	22	2270	4161	26	14856	37296	37	5836	29984	20	1144	1438	40	1037	948
37	2361	1141	51	2717	302	22	2306	4138	27	12974	35324	38	10211	23065	21	3519	2914	40	1163	1100

37	1973	954	52	2019	323	22	1701	2769	27	24760	57535	38	16511	36508	21	3568	2979	40	947	907
37	2079	1024	52	1448	257	22	1571	2515	27	23268	51851	38	15515	34086	21	3581	2971	40	1204	1200
38	8043	15752	52	1157	239	22	1732	2471	27	22032	48546	38	11102	24030	21	4337	3460	40	1295	1297
38	7038	13830	52	2027	484	22	1689	2371	27	27278	59018	38	11214	24048	22	1570	1255	40	1658	1679
38	6919	14358	52	1971	537	23	1209	1569	27	28492	55023	38	20102	42123	22	2692	2311	41	1337	971
38	6378	13849	54	1172	1270	23	884	1052	28	5141	9044	39	2262	8410	22	2945	2529	41	1488	1107
38	5379	13351	54	1068	1402	23	739	1119	28	5286	8536	39	3419	12660	22	3470	2977	41	1206	910
38	5073	13646	54	674	1045	24	4598	7452	28	4983	7755	39	3186	11484	22	1529	1192	41	1012	814
39	755	1306	54	896	1397	24	3533	5396	28	9157	14097	39	2618	9411	23	1949	1916	41	861	752
39	946	1646	55	229	2234	24	4014	6089	28	4805	7349	39	4092	14690	23	1397	1301	41	1085	972
39	973	1702	55	225	2403	24	2978	4258	28	9293	13840	39	3468	12274	23	2481	2326	42	1721	3757
39	917	1686	55	193	2173	24	3276	4582	29	3181	1982	40	1454	4319	24	4480	5526	42	1031	2266
39	720	1459	55	202	2376	24	4181	5793	29	3072	1894	40	1659	4660	24	3896	4386	42	2586	5701
39	768	1601	55	211	2543	26	3877	24928	29	3008	1683	40	1038	2751	24	5348	6397	42	2478	5556
40	1055	1889	55	166	2196	26	4467	28683	29	4884	2440	40	1200	3152	26	39189	9099	42	2207	4967
40	1013	1991	56	277	1297	26	3308	21191	30	6740	3971	40	1694	4448	26	49175	11679	42	2201	5095
40	819	1742	56	474	2254	26	4156	25509	30	7135	3754	40	1390	3409	26	40746	9701	43	275	965
40	678	1589	56	294	1634	26	3589	21964	30	4253	1695	43	2235	2187	27	40675	11193	43	319	1173
40	775	1936	56	441	2502	26	3124	18732	30	6803	2668	43	2367	2299	27	33921	9302	43	296	1169
40	480	1673	56	272	1695	27	2858	22121	30	5327	2052	43	2613	2440	27	28968	7527	43	250	1082
41	1298	1742	56	347	2239	27	3028	22896	30	7308	2575	43	4755	4367	27	56733	14710	46	1314	2538
41	1057	1732	57	1429	5248	27	3022	22631	31	4335	2355	46	2717	5055	27	46110	12051	46	1287	2621
41	1037	1733	57	1114	4506	27	1994	14605	31	6883	3386	46	5122	9253	27	43226	10865	46	2024	4172
41	758	1354	57	1045	4298	27	3158	23129	31	3965	1903	46	2046	3596	28	4224	3364	46	1708	3572
41	843	1511	57	747	3215	27	2344	17139	31	5261	2456	46	1500	2619	28	5100	3921	46	1832	3889
41	795	1535	57	1177	5404	28	1422	2537	31	6275	2831	47	7332	12800	28	3583	2582	46	2674	5700
42	1231	4428	57	818	4120	28	1282	2273	32	4332	2943	47	4711	7870	28	5564	4603	47	753	1777
42	1276	4678	58	428	1593	28	1589	2793	32	3839	2534	47	8501	13973	28	5031	4057	47	1394	3327
42	1261	4807	58	276	1078	28	1341	2322	32	1978	1305	47	5655	9223	28	5175	4198	47	1127	2699
42	1012	4238	58	399	1610	28	1086	1563	32	3907	2451	47	6335	10302	30	5500	1630	47	1256	3032
42	832	3523	58	305	1429	28	1044	1379	32	2938	1786	47	5138	8096	30	3524	1033	47	1013	2580
42	740	3962	63	1631	1383	29	1076	754	33	4352	2798	48	2313	4544	30	4788	1378	47	952	2582
46	1983	4560	63	685	709	29	1132	779	33	5270	3379	48	4925	9564	30	3794	1081	49	1279	2781
46	1712	4350	63	959	1132	29	709	345	33	3537	1972	48	2566	4787	30	5098	1486	49	933	2032
46	1389	3806	63	915	1261	30	1837	1589	33	2598	1431	48	3590	6694	30	5527	1638	49	859	1980
46	869	2879	65	1830	2865	30	1233	955	34	2400	1779	48	5869	10255	31	11344	3407	53	825	588
46	994	3610	65	1325	2135	30	1538	1058	34	3067	2137	49	13078	26006	31	10112	2943	53	977	743
46	694	2730	65	996	1638	30	1072	665	34	2254	1490	49	18455	32704	31	8271	2416	53	905	731
47	754	1481	65	1085	1830	31	2925	2485	34	3479	2161	49	10438	16094	31	6362	1794	53	1238	1001
47	1164	2302	65	910	1601	31	2650	2172	34	1883	1116	49	24119	35959	31	7991	2192	54	1413	1170
47	988	1963	84	1097	1135	31	1923	1434	34	2288	1293	50	43223	14334	31	12923	3570	54	1205	1021
47	1004	2042	84	958	1183	32	1217	974	36	1146	1553	51	18127	7444	32	1613	948	54	2397	2172
47	844	1718	84	598	853	32	902	526	36	1847	2402	51	13421	4925	32	1620	987	54	1853	1697
47	738	1684	84	902	1383	33	1172	1519	36	1047	1265	51	30267	10497	32	1498	827	55	846	4200
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50	9352	1594	85	1499	1453	33	729	796	37	4445	3215	52	19639	7128	33	3799	1407	55	1002	5109
50	10933	2173	85	2130	2132	33	710	759	37	5423	3825	62	2281	3755	33	6575	2807	56	371	1186
50	7077	1435	85	3567	3680	33	553	469	37	4119	2788	62	2574	4140	33	5749	2404	56	526	1775
50	7208	1465	85	2861	2963	34	990	1593	37	5141	3310	62	2876	4551	33	5506	2238	56	715	2503
50	12595	2628	85	2269	2441	34	1470	2294	37	4693	3001	62	2472	3736	33	3416	1399	57	500	1031
51	3282	506	86	1331	1038	34	1051	1395	37	3885	2428	62	1623	2376	33	4735	1806	57	1646	4417

51	2399	374	86	1240	1021	34	1115	1466	38	22367	65327	62	2899	4098	34	5651	2201	58	375	1053
51	4444	816	86	1258	1066	34	1029	1350	38	25231	64769	63	12905	18435	34	5909	2390	59	1308	12.12
51	2523	510	86	1608	1417	35	2156	2084	39	2924	8581	63	11345	16122	34	5931	2311	59	2366	402.4
52	1693	377	86	1892	1788	35	1559	1140	39	2646	7459	63	11665	16536	34	5312	1974	59	1763	223.16
52	2915	716	87	387	1743	35	1251	888	39	2428	6790	63	16567	23073	35	2838	2902	59	2012	236.7
52	3151	864	87	483	2238	35	1671	1168	39	2304	5779	63	16482	22649	35	2344	2252	59	1274	163.3
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72	4428	3427
73	6614	5329
73	6231	5113
73	4698	3706
73	1716	1787
73	1409	1525
75	1485	1450
75	2472	2381
75	1895	1905
75	2160	2133
75	2015	1567
75	2174	1573
76	2433	1874
76	1954	1483
76	2067	1551
76	4059	3112
76	2817	1993
77	2405	1700
77	5547	3699
77	4161	2911
77	8639	6906
77	7647	6095
78	8138	6319
78	7279	5373
78	9012	7112
78	1105	881
78	1270	956
79	1737	1404
79	1684	1437
80	1649	1354
80	1799	1484
80	1789	1461

98	3788	3148
99	1039	674
100	1232	1360
100	963	787
103	1232	836
103	1308	826
103	960	487
104	2691	6212
104	2289	5087
104	2324	4986
104	1821	3719
104	2078	4215
104	1271	2523
107	6738	6200
107	5150	4576
107	3163	2605
107	4578	3737
107	3389	2745
107	2029	1297
108	2323	1463
109	5118	8158
109	3320	4606
109	5700	7903
109	3416	4600
109	4647	6073
109	5238	6833
110	5612	3566
110	4441	2794
110	3704	1664
110	3474	1494
110	2957	1201
110	2685	887
111	10100	4784
111	5887	2282
111	6377	2450
111	5951	2229
111	6277	2301
111	8270	3004

91	704	2896
91	826	3097
91	593	2176
91	957	3305
91	697	2153
91	1045	3158
93	4733	26955
93	5499	30889
93	4817	26434
93	7165	38863
93	4417	23939
93	5773	30391
94	4388	8313
94	4865	9113
94	4959	9287
94	3701	6919
94	4898	8827
94	4609	8245
95	4907	9307
95	10720	20082
95	7761	14430
95	5796	10658
95	10393	19023
95	11486	19250
96	5250	1880
96	5929	2114
96	5368	1793
96	3773	1171
96	5863	1813
96	6030	1796
97	2277	6563
97	2293	6063
97	4432	11382
97	2773	6670
97	3617	8124
98	4330	9861
98	4337	9528
98	5664	12346
98	7080	15317
98	6533	13765
104	2430	7531
104	3443	10557
104	1796	5420
104	2998	8539
104	2484	6981
106	5068	2685
106	9251	4799
106	10106	5175
106	9379	4655
106	5014	2468
108	5265	4915

80	1692	1259
80	1204	720
81	2240	1029
82	1864	819
83	3899	1891
83	3019	1435
83	2550	1112
83	2189	994
83	11063	8314
83	5779	4280
84	6617	5401
84	5434	4201
84	26591	18972
84	28297	20075
85	17977	12613
85	26318	17953
85	28065	19403
85	33018	21847
85	9661	7633
85	11706	9280
86	7820	5985
86	6643	5178
86	10406	8142
86	10895	8337
86	5858	18008
86	5953	18200
87	6112	19268
87	4265	12785
87	5017	14838
87	5921	17667
87	4759	9740
87	6514	13447
88	7346	15611
88	3721	7173
88	4682	9183
88	4957	9984
88	7489	11261
88	3984	5184
93	4333	6080
93	6030	8559
93	5178	7006
93	7286	10039
93	957	8425
93	786	6060
94	601	4335
94	709	5705
94	983	7743
94	1150	9759
94	560	5568
94	1025	10026
95	451	3715

108	3580	3335
108	4212	3799
108	3684	3051
108	3458	2812
109	4941	13704
109	4121	11072
109	4938	13002
109	3848	10019
109	7677	19216
110	6854	3621
110	5314	2804
110	3562	1833
110	4981	2539
110	3860	1859
95	673	6030
95	502	4504
95	735	7121
95	7398	9146
95	7358	9030
97	11993	14814
97	10416	12941
97	10515	12981
97	8092	9735
97	12660	13751
97	7919	8505
98	14272	16662
98	11447	13420
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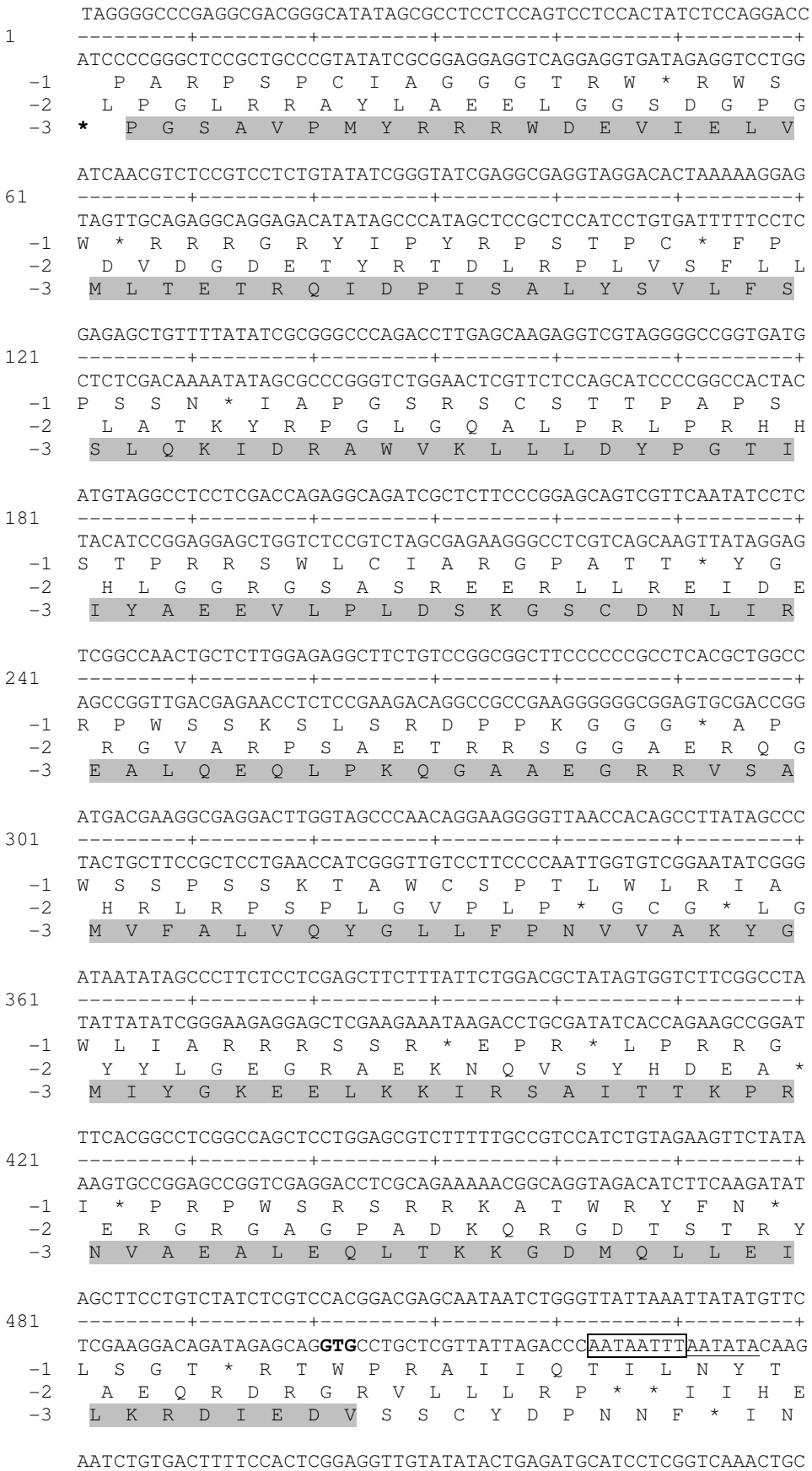
98	12763	14133
98	25890	5798
98	15141	3552
104	11433	2682
104	9691	1921
104	13155	2856
105	13902	2798
105	13829	2940
105	13382	2694
105	9573	1808
105	31069	5785
105	39821	8447
106	24722	5244
106	27505	5706
106	27056	5434
106	23933	4654
106	28069	2548
106	21789	1934
107	35334	3228
107	38867	3664
107	19285	1516
107	5421	6623
107	5689	6883
109	2716	3092
109	3331	3863
109	3840	4571
109	7649	2947
109	4601	1822
110	4321	1646
110	4626	1756
110	2829	1452
110	3501	1796
111	2975	1460
111	3484	1779
111	3246	1539
111	2869	1439

* Normalised intensities

Fig. A1 Nucleotide- and corresponding amino acid sequence of the *T. tenax* Lrp1and HP5



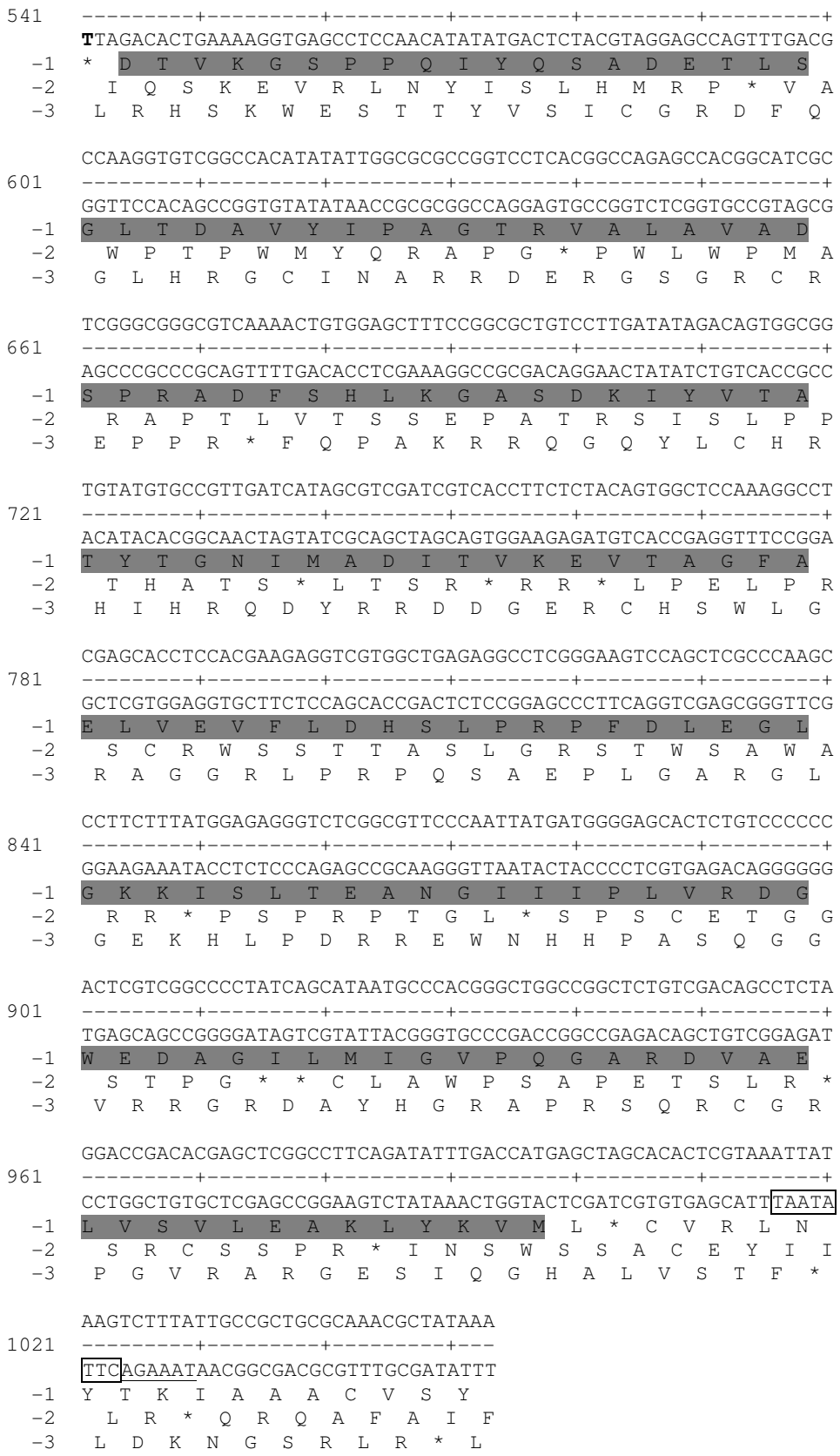
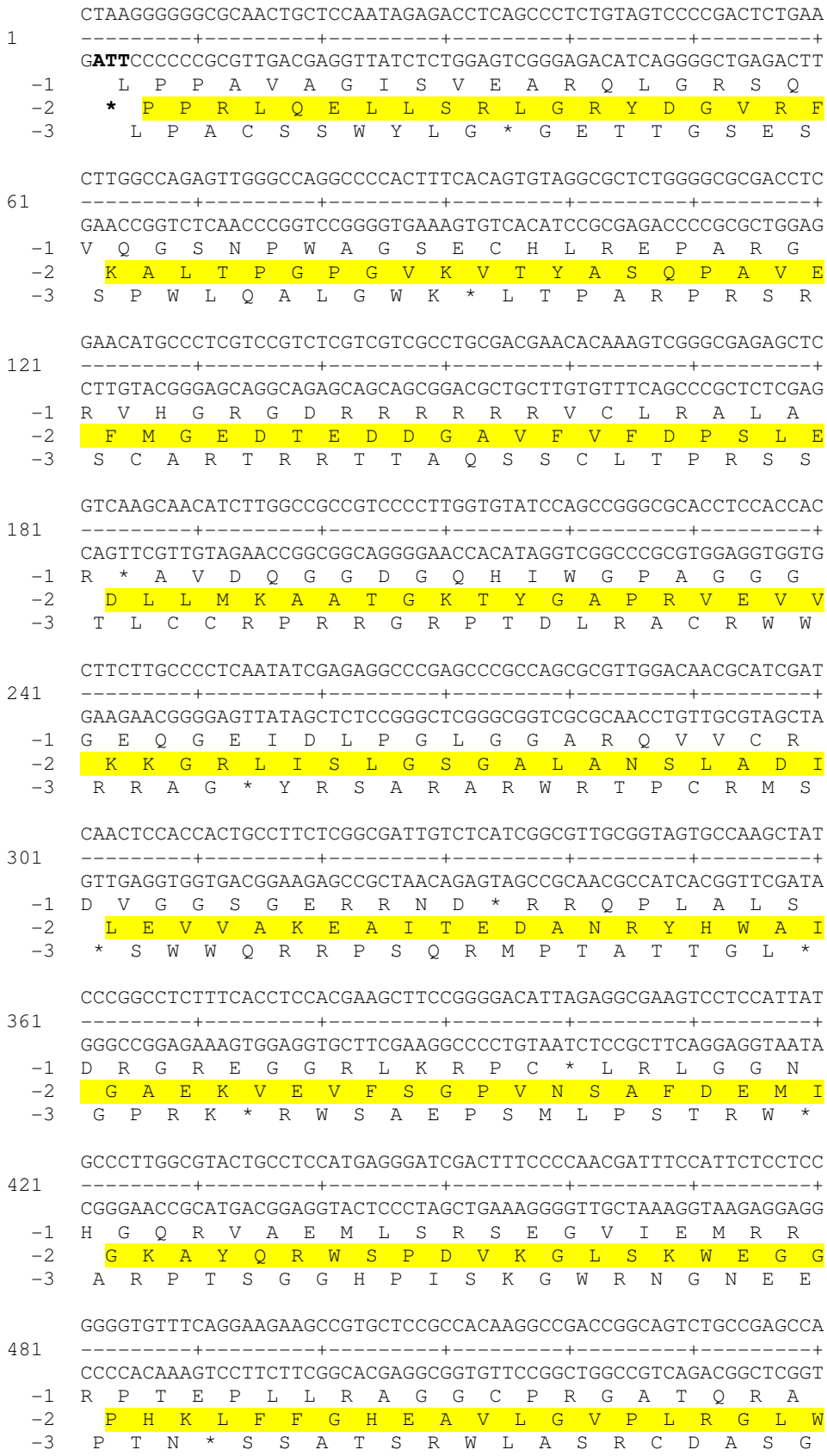


Fig. A1 **Nucleotide- and corresponding amino acid sequences of the *T. tenax lrp1* and *hp5* gene.** The *lrp1* gene (TTX_1154, 501 bp; shaded light grey) encodes the leucine-responsive regulatory protein (Lrp1) and *hp5* (TTX_1155, 459 bp; dark grey) is coding for hypothetical protein5. The start codons (bold), stop codons (asterisk) and putative BRE site (underlined) and TATA-Box (boxed) are marked.

Fig. A2 **Nucleotide- and corresponding amino acid sequence of the *T. tenax* trehalose operon**



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      GCCGTCCAGAAAGTCTCTGCCCCTCCCGCTCACTACGTAGACCGCCGTCTCGGGGAGCGC
541   ---------+---------+---------+---------+---------+---------+
      CGGCAGGTCTTTCAGAGACGGGGAGGGCGAGTGATGCATCTGGCGGCAGAGCCCCTCGCG
  -1  L  R  G  S  L  R  Q  G  E  R  E  S  R  L  G  G  D  R  P  A
  -2    G  D  L  F  D  R  G  R  G  S  V  V  Y  V  A  T  E  P  L  A
  -3   A  T  W  F  T  E  A  G  G  A  *  *  T  S  R  R  R  P  S  R

      CGCAAGGCGGCTCAACAGCTCCAACAGATCGCCGTCTGGAACCGCCATGTGCGGGTAGGG
601   ---------+---------+---------+---------+---------+---------+
      GCGTTCCGCCGAGTTGTCGAGGTTGTCTAGCGGCAGACCTTGGCGGTACACGCCCATCCC
  -1  G  C  P  P  E  V  A  G  V  S  R  R  R  S  G  G  H  A  P  L
  -2    A  L  R  S  L  L  E  L  L  D  G  D  P  V  A  M  H  P  Y  P
  -3   R  L  A  A  *  C  S  W  C  I  A  T  Q  F  R  W  T  R  T  P

      ATAGTGGGGGACCAACGTGCCGTCGTAGTCCAAGAGCAAGAGCCTCCTCTTGGCCTTGGC
661   ---------+---------+---------+---------+---------+---------+
      TATCACCCCCTGGTTGCACGGCAGCATCAGGTTCTCGTTCTCGGAGGAGAACCGGAACCG
  -1  S  L  P  P  G  V  H  R  R  L  G  L  A  L  A  E  E  Q  G  Q
  -2    Y  H  P  V  L  T  G  D  Y  D  L  L  L  L  R  R  K  A  K  A
  -3   I  T  P  S  W  R  A  T  T  T  W  S  C  S  G  G  R  P  R  P

      GAAGTCAGACTTGAGTTTGTCCACCGAGAGCTCCTCCATATATCTGAGGGCCTTCTCCAC
721   ---------+---------+---------+---------+---------+---------+
      CTTCAGTCTGAACTCAAACAGGTGGCTCTCGAGGAGGTATATAGACTCCCGGAAGAGGTG
  -1  R  L  *  V  Q  T  Q  G  G  L  A  G  G  Y  I  Q  P  G  E  G
  -2    F  D  S  K  L  K  D  V  S  L  E  E  M  Y  R  L  A  K  E  V
  -3   S  T  L  S  S  N  T  W  R  S  S  R  W  I  D  S  P  R  R  W

      CTCCTCTCGGGCCGACTTCGCCGAGATTAGGGAGTAGATGAAGTCGGTGCCCCATCTGAC
781   ---------+---------+---------+---------+---------+---------+
      GAGGAGAGCCCGGCTGAAGCGGCTCTAATCCCTCATCTACTTCAGCCACGGGGTAGACTG
  -1  G  G  R  P  G  V  E  G  L  N  P  L  L  H  L  R  H  G  M  Q
  -2    E  E  R  A  S  K  A  S  I  L  S  Y  I  F  D  T  G  W  R  V
  -3   R  R  E  P  R  S  R  R  S  *  P  T  S  S  T  P  A  G  D  S

      CACGTCCCTCATCCTCAGCCTCTCCTGCATAGCCCTGATCCTCCTACACTGCTCGTCCTC
841   ---------+---------+---------+---------+---------+---------+
      GTGCAGGGAGTAGGAGTCGGAGAGGACGTATCGGGACTAGGAGGATGTGACGAGCAGGAG
  -1  G  R  G  E  D  E  A  E  G  A  Y  G  Q  D  E  *  V  A  R  G
  -2    V  D  R  M  R  L  R  E  Q  M  A  R  I  R  R  C  Q  E  D  E
  -3   W  T  G  *  G  *  G  R  R  C  L  G  S  G  G  V  S  S  T  R

      CGACATGGAGAGCGCCTCCGCTATCGCCTCCGCAGTGCCCCCCACGTCGTTTGGGTTTAT
901   ---------+---------+---------+---------+---------+---------+
      GCTGTACCTCTCGCGGAGGCGATAGCGGAGGCGTCACGGGGGGTGCAGCAAACCCAAATA
  -1  G  V  H  L  A  G  G  S  D  G  G  C  H  G  G  R  R  K  P  K
  -2    S  M  S  L  A  E  A  I  A  E  A  T  G  G  V  D  N  P  N  I
  -3   R  C  P  S  R  R  R  *  R  R  R  L  A  G  W  T  T  Q  T  *

      CACCAACGCCTCGGCCAGCTCCTTGGAGGCGCCGGCCAGTTCGCTGAGTATAAGCACTCC
961   ---------+---------+---------+---------+---------+---------+
      GTGGTTGCGGAGCCGGTCGAGGAACCTCCGCGGCCGGTCAAGCGACTCATATTCGTGAGG
  -1  D  G  V  G  R  G  A  G  Q  L  R  R  G  T  R  Q  T  Y  A  S
  -2    V  L  A  E  A  L  E  K  S  A  G  A  L  E  S  L  I  L  V  G
  -3   *  W  R  R  P  W  S  R  P  P  A  P  W  N  A  S  Y  L  C  E

      GCGGCAGTCCCTCTTGGAGGCCACGAACTCCTTCGCCACCAAGTTCATTCCGTCGCGCAG
1021  ---------+---------+---------+---------+---------+---------+
      CGCCGTCAGGGAGAACCTCCGGTGCTTGAGGAAGCGGTGGTTCAAGTAAGGCAGCGCGTC
  -1  R  P  L  G  E  Q  L  G  R  V  G  E  G  G  L  E  N  R  R  A
  -2    R  C  D  R  K  S  A  V  F  E  K  A  V  L  N  M  G  D  R  L
  -3   A  A  T  G  R  P  P  W  S  S  R  R  W  W  T  *  E  T  A  C

      CGGAGTTATCAGAGCAACGTCGGCTATATTGTACAGCGCCATGAGGGTGGGCGATGGGAT
1081  ---------+---------+---------+---------+---------+---------+
      GCCTCAATAGTCTCGTTGCAGCCGATATAACATGTCGCGGTACTCCCACCCGCTACCCTA
  -1  A  S  N  D  S  C  R  R  S  Y  Q  V  A  G  H  P  H  A  I  P
  -2    P  T  I  L  A  V  D  A  I  N  Y  L  A  M  L  T  P  S  P  I
  -3   R  L  *  *  L  L  T  P  *  I  T  C  R  W  S  P  P  R  H  S
```

```
      GAAGCGGTACAAGTACACTATAGGGACCCAGTTGAGCTCCCCCAGCTCCCCATTTATTCT
1141  ---------+---------+---------+---------+---------+---------+
      CTTCGCCATGTTCATGTGATATCCCTGGGTCAACTCGAGGGGGTCGAGGGGTAAATAAGA
  -1  H  L  P  V  L  V  S  Y  P  G  L  Q  A  G  G  A  G  W  K  N
  -2     F  R  Y  L  Y  V  I  P  V  W  N  L  E  G  L  E  G  N  I  R
  -3   S  A  T  C  T  C  *  L  S  G  T  S  S  G  W  S  G  M  *  E

      GCCGACCTCTCTGTCTATCTGCCTCTTCATTTCCTCGTACATGGGCACGCCCGTCCTCGA
1201  ---------+---------+---------+---------+---------+---------+
      CGGCTGGAGAGACAGATAGACGGAGAAGTAAAGGAGCATGTACCCGTGCGGGCAGGAGCT
  -1  Q  R  G  R  Q  R  D  A  E  E  N  G  R  V  H  A  R  G  D  E
  -2     G  V  E  R  D  I  Q  R  K  M  E  E  Y  M  P  V  G  T  R  S
  -3   A  S  R  E  T  *  R  G  R  *  K  R  T  C  P  C  A  R  G  R

      GGGCACGACCACCAACACGAACACGGCTCTGCCGCGCCACTCGGGGTGCTCCCTCAAGAA
1261  ---------+---------+---------+---------+---------+---------+
      CCCGTGCTGGTGGTTGTGCTTGTGCCGAGACGGCGCGGTGAGCCCCACGAGGGAGTTCTT
  -1  L  A  R  G  G  V  R  V  R  S  Q  R  A  V  R  P  A  G  E  L
  -2     P  V  V  V  L  V  F  V  A  R  G  R  W  E  P  H  E  R  L  F
  -3   P  C  S  W  W  C  S  C  P  E  A  A  G  S  P  T  S  G  *  S

      CCTCTCCCACGCGGCCACCCTCCTTAGGACGCCCTTAGTGTAGTCAAGTCTGTCTATAGA
1321  ---------+---------+---------+---------+---------+---------+
      GGAGAGGGTGCGCCGGTGGGAGGAATCCTGCGGGAATCACATCAGTTCAGACAGATATCT
  -1  V  E  G  V  R  G  G  E  K  P  R  G  *  H  L  *  T  Q  R  Y
  -2     R  E  W  A  A  V  R  R  L  V  G  K  T  Y  D  L  R  D  I  S
  -3   G  R  G  R  P  W  G  G  *  S  A  R  L  T  T  L  D  T  *  L

      GAACACAACCTTCGCGCGCCCCAACATCTCTCTGAGCTTCGCCATCTCCTCGACTACAGA
1381  ---------+---------+---------+---------+---------+---------+
      CTTGTGTTGGAAGCGCGCGGGGTTGTAGAGAGACTCGAAGCGGTAGAGGAGCTGATGTCT
  -1  L  V  C  G  E  R  A  G  V  D  R  Q  A  E  G  D  G  R  S  C
  -2     F  V  V  K  A  R  G  L  M  E  R  L  K  A  M  E  E  V  V  S
  -3   S  C  L  R  R  A  G  W  C  R  E  S  S  R  W  R  R  S  *  L

      GGGATCCTGCGAGGAATTGTAGAATCTATCGAAGTCTATGCCTATGGGGAAGACGCCAAC
1441  ---------+---------+---------+---------+---------+---------+
      CCCTAGGACGCTCCTTAACATCTTAGATAGCTTCAGATACGGATACCCCTTCTGCGGTTG
  -1  L  S  G  A  L  F  Q  L  I  *  R  L  R  H  R  H  P  L  R  W
  -2     P  D  Q  S  S  N  Y  F  R  D  F  D  I  G  I  P  F  V  G  V
  -3   P  I  R  R  P  I  T  S  D  I  S  T  *  A  *  P  S  S  A  L

      TCTCACCCTTCTGTGGCCCACTGCGATGGCCCCCATCTCCACTTTGTATCCCAGGAATCT
1501  ---------+---------+---------+---------+---------+---------+
      AGAGTGGGAAGACACCGGGTGACGCTACCGGGGGGTAGAGGTGAAACATAGGGTCCTTAGA
  -1  S  E  G  K  Q  P  G  S  R  H  G  G  D  G  S  Q  I  G  P  I
  -2     R  V  R  R  H  G  V  A  I  A  G  M  E  V  K  Y  G  L  F  R
  -3   E  *  G  E  T  A  W  Q  S  P  G  W  R  W  K  T  D  W  S  D

      GACGACGCTCCTGGAGAAATTCGCGGAGTATTCGTATGTGTGGAAGCCGACGAGGTCCGA
1561  ---------+---------+---------+---------+---------+---------+
      CTGCTGCGAGGACCTCTTTAAGCGCCTCATAAGCATACACACCTTCGGCTGCTCCAGGCT
  -1  Q  R  R  E  Q  L  F  E  R  L  I  R  I  H  P  L  R  R  P  G
  -2     V  V  S  R  S  F  N  A  S  Y  E  Y  T  H  F  G  V  L  D  S
  -3   S  S  A  G  P  S  I  R  P  T  N  T  H  T  S  A  S  S  T  R

      CCCCAGGAGGCCCTCCAAGATCTCCCTCCGCCACTCGGAGGGCAGGAGCTGTAGAAGCTC
1621  ---------+---------+---------+---------+---------+---------+
      GGGGTCCTCCGGGAGGTTCTAGAGGGAGGCGGTGAGCCTCCCGTCCTCGACATCTTCGAG
  -1  V  G  P  P  G  G  L  D  G  E  A  V  R  L  A  P  A  T  S  A
  -2     G  L  L  G  E  L  I  E  R  R  W  E  S  P  L  L  Q  L  L  E
  -3   G  W  S  A  R  W  S  R  G  G  G  S  P  P  C  S  S  Y  F  S

      GGCCGGCGGGAAGGGGATGTGGAGGAAGAAGCCCACTCCGACCTCCGCCGCCTCTCTCAC
1681  ---------+---------+---------+---------+---------+---------+
      CCGGCCGCCCTTCCCCTACACCTCCTTCTTCGGGTGAGGCTGGAGGCGGCGGAGAGAGTG
  -1  R  G  A  P  L  P  H  P  P  L  L  G  S  R  G  G  G  G  R  E
  -2     A  P  P  F  P  I  H  L  F  F  G  V  G  V  E  A  A  E  R  V
  -3   P  R  R  S  P  S  T  S  S  S  A  W  E  S  R  R  R  R  E  *
```

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      TATCGCCGGCGCCAACATGAGGTGGTAGTCGTGGATCCACACTAGGTCGCCGGGGCGCGC
1741  ---------+---------+---------+---------+---------+---------+
      ATAGCGGCCGCGGTTGTACTCCACCATCAGCACCTAGGTGTGATCCAGCGGCCCCGCGCG
  -1  S  D  G  A  G  V  H  P  P  L  R  P  D  V  S  P  R  R  P  A
  -2     I  A  P  A  L  M  L  H  Y  D  H  I  W  V  L  D  G  P  R  A
  -3   *  R  R  R  W  C  S  T  T  T  T  S  G  C  *  T  A  P  A  R

      CAGAGCTACGACCGCTTTGGCGTACTTCTCGTTGACGCCTCTGTAGGCGCGCCAGTGCTT
1801  ---------+---------+---------+---------+---------+---------+
      GTCTCGATGCTGGCGAAACCGCATGAAGAGCAACTGCGGAGACATCCGCGCGGTCACGAA
  -1  G  S  S  R  G  S  Q  R  V  E  R  Q  R  R  Q  L  R  A  L  A
  -2     L  A  V  V  A  K  A  Y  K  E  N  V  G  R  Y  A  R  W  H  K
  -3   W  L  *  S  R  K  P  T  S  R  T  S  A  E  T  P  A  G  T  S

      CTCCTCATACGTCGCATACTCCGAGAAGCCGTGGAAGAGCGGCCAGAGAGTCGAATTGGA
1861  ---------+---------+---------+---------+---------+---------+
      GAGGAGTATGCAGCGTATGAGGCTCTTCGGCACCTTCTCGCCGGTCTCTCAGCTTAACCT
  -1  E  G  *  V  D  C  V  G  L  L  R  P  L  A  A  L  S  D  F  Q
  -2     E  E  Y  T  A  Y  E  S  F  G  H  F  L  P  W  L  T  S  N  S
  -3   R  R  M  R  R  M  S  R  S  A  T  S  S  R  G  S  L  R  I  P

      AAAGCCCTCGTAGAACCCCTCGACCTCCTCAGACGAGAGGGGGACAGGCTCTAGGCCCAT
1921  ---------+---------+---------+---------+---------+---------+
      TTTCGGGAGCATCTTGGGGAGCTGGAGGAGTCTGCTCTCCCCCTGTCCGAGATCCGGGTA
  -1  F  L  G  R  L  V  G  R  G  G  *  V  L  P  P  C  A  R  P  G
  -2     F  G  E  Y  F  G  E  V  E  E  S  S  L  P  V  P  E  L  G  M
  -3   F  A  R  T  S  G  R  S  R  R  L  R  S  P  S  L  S  *  A  W

      CCCTCTGAGGCGCTCCCTCAGATCGTTGGACTCCCTCTCCGAGGGCACGCCTGACCAGCC
1981  ---------+---------+---------+---------+---------+---------+
      GGGAGACTCCGCGAGGGAGTCTAGCAACCTGAGGGAGAGGCTCCCGTGCGGACTGGTCGG
  -1  D  R  Q  P  A  G  E  S  R  Q  V  G  E  G  L  A  R  R  V  L
  -2     G  R  L  R  E  R  L  D  N  S  E  R  E  S  P  V  G  S  W  G
  -3   G  E  S  A  S  G  *  I  T  P  S  G  R  R  P  C  A  Q  G  A

      GACCCAGACGACCTCCTCGAGGCCCAGCTCTCTGCCCCCGTTCACGGCGCCGAGGAAGGA
2041  ---------+---------+---------+---------+---------+---------+
      CTGGGTCTGCTGGAGGAGCTCCGGGTCGAGAGACGGGGGCAAGTGCCGCGGCTCCTTCCT
  -1  R  G  L  R  G  G  R  P  G  A  R  Q  G  R  E  R  R  R  P  L
  -2     V  W  V  V  E  E  L  G  L  E  R  G  G  N  V  A  G  L  F  S
  -3   S  G  S  S  R  R  S  A  W  S  E  A  G  T  *  P  A  S  S  P

      CTTCATAGCAGTCGCAAGGCCGCCCACGGACTCCCTTATCTCGCCGCTCGGCGATATAGT
2101  ---------+---------+---------+---------+---------+---------+
      GAAGTATCGTCAGCGTTCCGGCGGGTGCCTGAGGGAATAGAGCGGCGAGCCGCTATATCA
  -1  V  E  Y  C  D  C  P  R  G  R  V  G  K  D  R  R  E  A  I  Y
  -2     K  M  A  T  A  L  G  G  V  S  E  R  I  E  G  S  P  S  I  T
  -3   S  *  L  L  R  L  A  A  W  P  S  G  *  R  A  A  R  R  Y  L

      GACGGGCAATCTGTTGGAGACCACTATGAGGCGCACTTGTCCTCCCACGGAACGCCTTTT
2161  ---------+---------+---------+---------+---------+---------+
      CTGCCCGTTAGACAACCTCTGGTGATACTCCGCGTGAACAGGAGGGTGCCTTGCGGAAAA
  -1  H  R  A  I  Q  Q  L  G  S  H  P  A  S  T  R  G  R  F  A  K
  -2     V  P  L  R  N  S  V  V  I  L  R  V  Q  G  G  V  S  R  R  K
  -3   S  P  C  D  T  P  S  W  *  S  A  C  K  D  E  W  P  V  G  K

      TACGGTTGTGCCCCTAAAATTAATTCGGCGAGGCGCCCGATGCTCAGCTGAGGCGCCCTC
2221  ---------+---------+---------+---------+---------+---------+
      ATGCCAACACGGGGATTTTAATTAAGCCGCTCCGCGGGCTACGAGTCGACTCCGCGGGAG
  -1  K  R  N  H  G  *  F  *  N  P  S  A  G  S  A  *  S  L  R  G
  -2     V  T  T  G  R  F  N  I  R  R  P  A  R  H  E  A  S  A  G  E
  -3   *  P  Q  A  G  L  I  L  E  A  L  R  G  I  S  L  Q  P  A  R

      TCCTTGGCCTTGGCCCCCATCTCGGCCAACACATCCAGCCGCCTCACGAGGGACTCTATC
2281  ---------+---------+---------+---------+---------+---------+
      AGGAACCGGAACCGGGGGTAGAGCCGGTTGTGTAGGTCGGCGGAGTGCTCCCTGAGATAG
  -1  R  R  P  R  P  G  W  R  P  W  C  M  W  G  G  *  S  P  S  *
  -2     G  Q  G  Q  G  G  D  R  G  V  C  G  A  A  E  R  P  V  R  D
  -3   E  K  A  K  A  G  M  E  A  L  V  D  L  R  R  V  L  S  E  I
```

```
     ACTCTGGGCGCGTCGCCCAGTTGGGCGAGCCTAACTCCGTCTACGCCGTCTCTGACCAAC
2341 ---------+---------+---------+---------+---------+---------+
     TGAGACCCGCGCAGCGGGTCAACCCGCTCGGATTGAGGCAGATGCGGCAGAGACTGGTTG
  -1 *  E  P  R  T  A  W  N  P  S  G  L  E  T  *  A  T  E  S  W
  -2    S  Q  A  R  R  G  T  P  R  A  *  S  R  R  R  R  R  Q  G  V
  -3    V  R  P  A  D  G  L  Q  A  L  R  V  G  D  V  G  D  R  V  L

     CTCGAGGCGTGGTTGTACTGCGAGACCGCGACGGGCACTCCCAGGCCCATGAACTCCAAC
2401 ---------+---------+---------+---------+---------+---------+
     GAGCTCCGCACCAACATGACGCTCTGGCGCTGCCCGTGAGGGTCCGGGTACTTGAGGTTG
  -1 G  R  P  T  T  T  S  R  S  R  S  P  C  E  W  A  W  S  S  W
  -2    E  L  R  P  Q  V  A  L  G  R  R  A  S  G  P  G  H  V  G  V
  -3    R  S  A  H  N  Y  Q  S  V  A  V  P  V  G  L  G  M  F  E  L

     ACGCGAATGCCCGCCGGCTCGTAGGAGGAGAGGTCGACTCCCAACGCGGACTTCGCCATC
2461 ---------+---------+---------+---------+---------+---------+
     TGCGCTTACGGGCGGCCGAGCATCCTCCTCTCCAGCTGAGGGTTGCGCCTGAAGCGGTAG
  -1 C  A  F  A  R  R  S  T  P  P  S  T  S  E  W  R  P  S  R  W
  -2    R  S  H  G  G  A  R  L  L  L  P  R  S  G  V  R  V  E  G  D
  -3    V  R  I  G  A  P  E  Y  S  S  L  D  V  G  L  A  S  K  A  M

     ACCGGCTCGGGCTCCACGTCCTCAAGGACCAAGAGGTTCTTGGGCCGGGCCCTCTTGAGT
2521 ---------+---------+---------+---------+---------+---------+
     TGGCCGAGCCCGAGGTGCAGGAGTTCCTGGTTCTCCAAGAACCCGGCCCGGGAGAACTCA
  -1 *  R  S  P  S  W  T  R  L  S  W  S  T  R  P  G  P  G  R  S
  -2    G  A  R  A  G  R  G  *  P  G  L  P  E  Q  A  P  G  E  Q  T
  -3    V  P  E  P  E  V  D  E  L  V  L  L  N  K  P  R  A  R  K  L

     CGCTGTGCCTCGCCCCCAGCGCCTACGGCCGCTACGACGATGTTGCCCGCCGAGGAGAGC
2581 ---------+---------+---------+---------+---------+---------+
     GCGACACGGAGCGGGGGTCGCGGATGCCGGCGATGCTGCTACAACGGGCGGCTCCTCTCG
  -1 D  S  H  R  A  G  L  A  *  P  R  *  S  S  T  A  R  R  P  S
  -2    A  T  G  R  G  W  R  R  R  G  S  R  R  H  Q  G  G  L  L  A
  -3    R  Q  A  E  G  G  A  G  V  A  A  V  V  I  N  G  A  S  S  L

     CGCTCGGCCGTCTTGGCCAACAGCTCTAAGTTTTTGTCCTCCTCAAGCGGCCCAACGTAG
2641 ---------+---------+---------+---------+---------+---------+
     GCGAGCCGGCAGAACCGGTTGTCGAGATTCAAAAACAGGAGGAGTTCGCCGGGTTGCATC
  -1 G  S  P  R  R  P  W  C  S  *  T  K  T  R  R  L  R  G  L  T
  -2    A  R  G  D  Q  G  V  A  R  L  K  Q  G  G  *  A  A  W  R  L
  -3    R  E  A  T  K  A  L  L  E  L  N  K  D  E  E  L  P  G  V  Y

     GCCACCACGGCCTCCGCTCCCTCTAGCTCGCCCGGAGGAGGGCGGCTCGCGCGGGCCAAC
2701 ---------+---------+---------+---------+---------+---------+
     CGGTGGTGCCGGAGGCGAGGGAGATCGAGCGGGCCTCCTCCCGCCGAGCGCGCCCGGTTG
  -1 P  W  W  P  R  R  E  R  *  S  A  R  L  L  A  A  R  A  P  W
  -2    G  G  R  G  G  S  G  R  A  R  G  S  S  P  P  E  R  P  G  V
  -3    A  V  V  A  E  A  G  E  L  E  G  P  P  P  R  S  A  R  A  L

     AGCTCTTCGTCGATCCAATTGGGGGCCACCGCGATCCTCCGCGGCCTATACTGCGACAGC
2761 ---------+---------+---------+---------+---------+---------+
     TCGAGAAGCAGCTAGGTTAACCCCCGGTGGCGCTAGGAGGCGCCGGATATGACGCTGTCG
  -1 C  S  K  T  S  G  I  P  P  W  R  S  G  G  R  G  I  S  R  C
  -2    A  R  R  R  D  L  Q  P  G  G  R  D  E  A  A  *  V  A  V  A
  -3    L  E  E  D  I  W  N  P  A  V  A  I  R  R  P  R  Y  Q  S  L

     TCCTCGGCCTCCTCCTCGCTCATTGCCACAACTAAATCTGCAGCGCGGATCACGCGGGGG
2821 ---------+---------+---------+---------+---------+---------+
     AGGAGCCGGAGGAGGAGCGAGTAACGGTGTTGATTTAGACGTCGCGCCTAGTGCGCCCCC
  -1 S  R  P  R  R  R  A  *  Q  W  L  *  I  Q  L  A  S  *  A  P
  -2    G  R  G  G  G  R  E  N  G  C  S  F  R  C  R  P  D  R  P  P
  -3    E  E  A  E  E  E  S  M  A  V  V  L  D  A  A  R  I  V  R  P

     CAGTGCAAGGCGCACCACATGAGCTTCGAGGCGCCCAGGAGGGACCGGGCGCTCGGCGGA
2881 ---------+---------+---------+---------+---------+---------+
     GTCACGTTCCGCGTGGTGTACTCGAAGCTCCGCGGGTCCTCCCTGGCCCGCGAGCCGCCT
  -1 A  T  C  P  A  G  C  S  S  R  P  A  W  S  P  G  P  A  R  R
  -2    L  A  L  R  V  V  H  A  E  L  R  G  P  P  V  P  R  E  A  S
  -3    C  H  L  A  C  W  M  L  K  S  A  G  L  L  S  R  A  S  P  P
```

```
      GGCGGCAGATACAGCGGTATGTATACTACGGTCACGCCTCTGGCCTCGCCCAGAGCAGCG
2941  ---------+---------+---------+---------+---------+---------+
      CCGCCGTCTATGTCGCCATACATATGATGCCAGTGCGGAGACCGGAGCGGGTCTCGTCGC
  -1  L  R  C  I  C  R  Y  T  Y  *  P  *  A  E  P  R  A  W  L  L
  -2     A  A  S  V  A  T  H  I  S  R  D  R  R  Q  G  R  G  S  C  R
  -3     P  P  L  Y  L  P  I  Y  V  V  T  V  G  R  A  E  G  L  A  A

      AGGGACCTTCTGACCTCAGCCCAGCGCGCTACCTCCTCGGGCCCGTTCCAGAAGCTCGAG
3001  ---------+---------+---------+---------+---------+---------+
      TCCCTGGAAGACTGGAGTCGGGTCGCGCGATGGAGGAGCCCGGGCAAGGTCTTCGAGCTC
  -1  S  P  G  E  S  R  L  G  A  R  *  R  R  P  G  T  G  S  A  R
  -2     P  V  K  Q  G  *  G  L  A  S  G  G  R  A  R  E  L  L  E  L
  -3     L  S  R  R  V  E  A  W  R  A  V  E  E  P  G  N  W  F  S  S

      AGGACGACGACTACGTCGAAGCCCAGTACGTCGTCTATGTTCCTCAAAATCGAGGGGAAG
3061  ---------+---------+---------+---------+---------+---------+
      TCCTGCTGCTGATGCAGCTTCGGGTCATGCAGCAGATACAAGGAGTTTTAGCTCCCCTTC
  -1  S  S  S  S  *  T  S  A  W  Y  T  T  *  T  G  *  F  R  P  S
  -2     P  R  R  S  R  R  L  G  T  R  R  R  H  E  E  F  D  L  P  L
  -3     L  V  V  V  V  D  F  G  L  V  D  D  I  N  R  L  I  S  P  F

      CTCCTGAGGGAGATGCTTCCTGACAGAGACCTCTGGCTCAGTACTCTGATTGTCGGAACC
3121  ---------+---------+---------+---------+---------+---------+
      GAGGACTCCCTCTACGAAGGACTGTCTCTGGAGACCGAGTCATGAGACTAACAGCCTTGG
  -1  A  G  S  P  S  A  E  Q  C  L  G  R  A  *  Y  E  S  Q  R  F
  -2     E  Q  P  L  H  K  R  V  S  V  E  P  E  T  S  Q  N  D  S  G
  -3     S  R  L  S  I  S  G  S  L  S  R  Q  S  L  V  R  I  T  P  V

      CCCGAGGGGTCCTCCTTGAGCGCCACGTAGCCGCCCAGACTCCTCTCCAGTACCTCCTCG
3181  ---------+---------+---------+---------+---------+---------+
      GGGCTCCCCAGGAGGAACTCGCGGTGCATCGGCGGGTCTGAGGAGAGGTCATGGAGGAGC
  -1  G  R  P  T  R  R  S  R  W  T  A  A  W  V  G  R  W  Y  R  R
  -2     G  L  P  G  G  Q  A  G  R  L  R  G  S  E  E  G  T  G  G  R
  -3     G  S  P  D  E  K  L  A  V  Y  G  G  L  S  R  E  L  V  E  E

      TCCGCTGCGGGCTCTCCCTCGTGGTATACGCTGGTTATCAGCCAGGCCTTGGCATATCTG
3241  ---------+---------+---------+---------+---------+---------+
      AGGCGACGCCCGAGAGGGAGCACCATATGCGACCAATAGTCGGTCCGGAACCGTATAGAC
  -1  T  R  Q  P  S  E  R  T  T  Y  A  P  *  *  G  P  R  P  M  D
  -2     G  S  R  A  R  G  R  P  I  R  Q  N  D  A  L  G  Q  C  I  Q
  -3     D  A  A  P  E  G  E  H  Y  V  S  T  I  L  W  A  K  A  Y  R

      TTGAGCGCCCTCACTAACAGAGCCCCGGCCCTATCGGCGTCCCTCCAACGGGAGGTCTGC
3301  ---------+---------+---------+---------+---------+---------+
      AACTCGCGGGAGTGATTGTCTCGGGGCCGGGATAGCCGCAGGGAGGTTGCCCTCCAGACG
  -1  T  S  R  G  *  *  C  L  G  P  G  I  P  T  G  G  V  P  P  R
  -2     Q  A  G  E  S  V  S  G  R  G  *  R  R  G  E  L  P  L  D  A
  -3     N  L  A  R  V  L  L  A  G  A  R  D  A  D  R  W  R  S  T  Q

      GGCGCCACTACAGCTACGTTCATCTTTTGAGGAGCCGGGCGATCACGACGGCGACCATGG
3361  ---------+---------+---------+---------+---------+---------+
      CCGCGGTGATGTCGATGCAAGTAGAAAACTCCTCGGCCCGCTAGTGCTGCCGCTGGTACC
  -1  R  R  W  *  L  *  T  *  R  K  L  L  R  A  I  V  V  A  V  M
  -2     A  G  S  C  S  R  E  D  K  S  S  G  P  S  *  S  P  S  W  P
  -3     P  A  V  V  A  V  N  M  K  Q  P  A  P  R  D  R  R  R  G  H

      CCGCGACCAGGCCCCACGCGATAGGCATAAGCATGGCGCTCAGTATCGAGACGTCTATGC
3421  ---------+---------+---------+---------+---------+---------+
      GGCGCTGGTCCGGGGTGCGCTATCCGTATTCGTACCGCGAGTCATAGCTCTGCAGATACG
  -1  A  A  V  L  G  W  A  I  P  M  L  M  A  S  L  I  S  V  D  I
  -2     R  S  W  A  G  R  S  L  C  L  C  P  A  *  Y  R  S  T  *  A
  -3     G  R  G  P  G  V  R  Y  A  Y  A  H  R  E  T  D  L  R  R  H

      CGCCCTGTTGGAGGGCGAAGGTGACCACCACGAGGTAGAGCACTATCCTGAGGTAGTCCG
3481  ---------+---------+---------+---------+---------+---------+
      GCGGGACAACCTCCCGCTTCCACTGGTGGTGCTCCATCTCGTGATAGGACTCCATCAGGC
  -1  G  G  Q  Q  L  A  F  T  V  V  V  L  Y  L  V  I  R  L  Y  D
  -2     A  R  N  S  P  S  P  S  W  W  S  T  S  C  *  G  S  T  T  R
  -3     R  G  T  P  P  R  L  H  G  G  R  P  L  A  S  D  Q  P  L  G
```

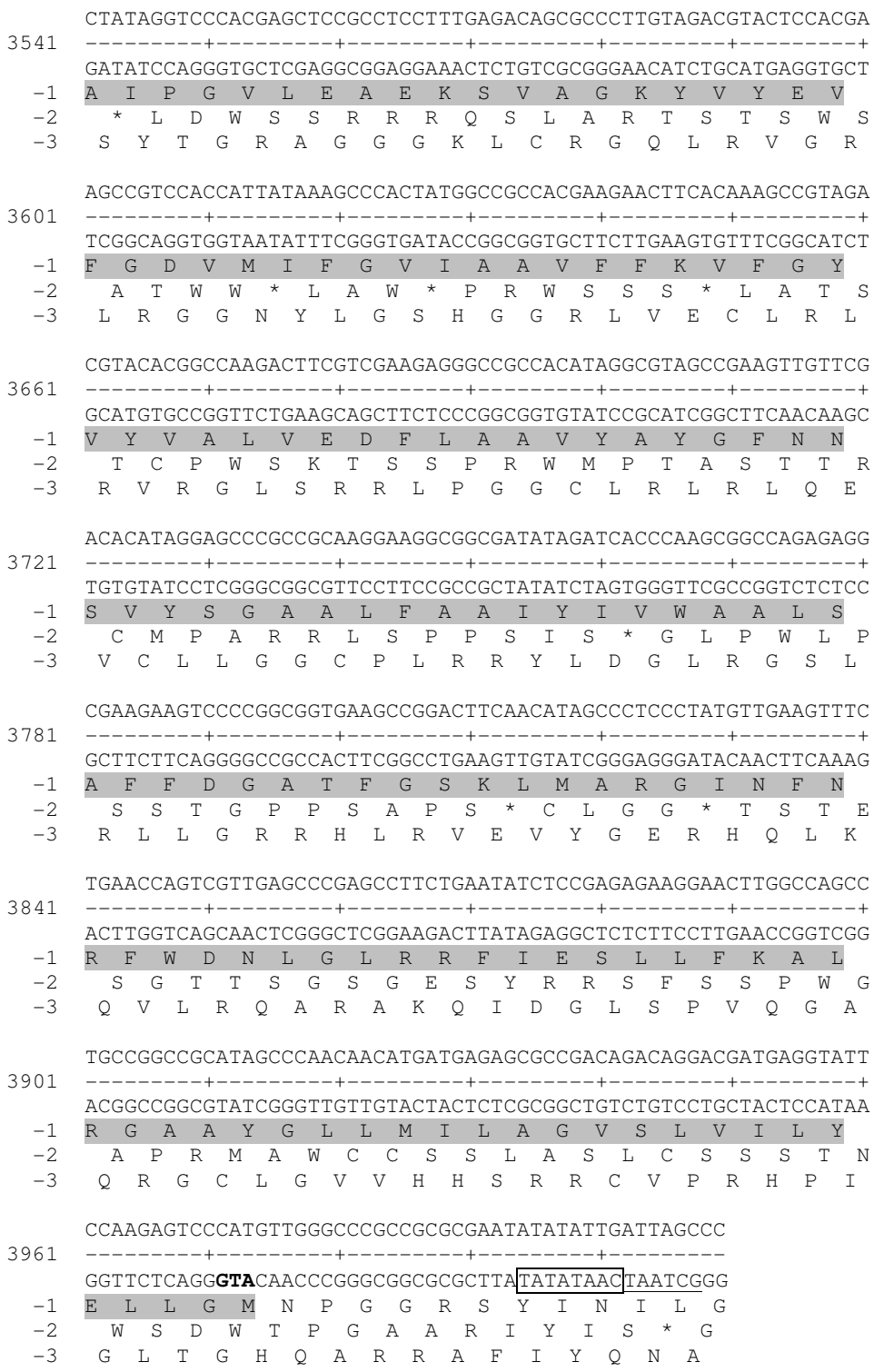

Fig. A2 **Nucleotide- and corresponding amino acid sequences of the *T. tenax msc, gt* and *tpsp* gene of the trehalose operon.** The *T. tenax* trehalose operon comprises the genes coding for trehalose-6-phosphate synthase/phosphatase (TTX_1304; *tpsp*; TPSP; shaded black), putative glycosyl transferase (TTX_1305; *gt*; GT; shaded dark grey) and the putative mechanosensitive channel (TTX_1304a; *msc*; MscTTX; shaded light grey). The start codons (bold), stop codons (asterisk) and putative BRE site (underlined) and TATA-Box (boxed) are marked.

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ERKLÄRUNG

Hiermit erkläre ich, gemäß § 6 Abs. 2, Nr. 6 der Promotionsordnung der Math.-Nat. Fachbereiche zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmitteln bedient habe.

Essen, den 02.04. 2007

Melanie Zaparty

ERKLÄRUNG

Hiermit erkläre ich, gemäß § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat. Fachbereiche zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Transcriptional regulation of the central carbohydrate metabolism and synthesis of trehalose in the hyperthermophilic crenarchaeote *Thermoproteus tenax*“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Frau Melanie Zaparty befürworte.

Essen, den 02.04. 2007

Prof. Dr. Reinhard Hensel

ERKLÄRUNG

Hiermit erkläre ich, gemäß § 6 Abs. 2, Nr. 8 der Promotionsordnung der Math.-Nat. Fachbereiche zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, den 02.04. 2007

Melanie Zaparty

LEBENSLAUF

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Betreuerin	HD Dr. Bettina Siebers, Universität Duisburg-Essen
Thema	„Untersuchungen zur Bedeutung von Trehalose und deren Synthese im hyperthermophilen Crearchaeum *Thermoproteus tenax*“

Promotionsarbeit

Zeitraum	März 2003 bis April 2007
Betreuer	Prof. Dr. Reinhard Hensel
Thema	‘‘Transcriptional regulation of the central carbohydrate metabolism and synthesis of trehalose in the hyperthermophilic crenarchaeote *Thermoproteus tenax”*